ETIOLOGICAL EVALUATION OF ATYPICAL COPPER RELATED LIVER DISEASE

A dissertation submitted in part fulfillment of the requirements for DM (Branch IV , Gastroenterology) examination of the Tamil Nadu Dr.M.GR. Medical University, Chennai to be held in August 2009

Certificate

This is to certify that this dissertation entitled 'Etiological Evaluation of Atypical copper related liver disease' is a bonafide work done by Dr.Y. Pavan Kumar Reddy in partial fulfillment of rules and regulations for DM (Branch IV-Gastroenterology) examination of the Tamil Nadu Dr. MGR Medical University, to be held in August 2009.

Guide

Dr.George M. Chandy
MD, DM, PGDHA ,FRCP,FIHS
Professor, Department of GI Sciences
Christian Medical College, Vellore

Head of the department

B.S Ramakrishna
MD, DM, PhD, FAMS
Professor, Department of GI Sciences
Christian Medical College, Vellore

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INTRODUCTION

Copper related liver disease is one of the most common etiologies of liver disease in India.

Wilsons disease (WD) is the most common of the copper related liver diseases. It is a genetic disorder which follows autosomal recessive inheritance. WD is characterized by a mutation in ATP 7B gene at 13 q 14. WD generally presents as persistently elevated aspartate / alanine aminotranferases , chronic hepatitis, compensated or decompensated chronic liver disease or rarely it may present as Acute Wilsonian crisis. WD is diagnosed by Serum Ceruloplasmin levels, KF ring, 24 hr urinary copper level, hepatic copper quantification and / or ATP 7 B mutation analysis. WD is treated by copper chelation either with Pencillamine or Zinc or both.

Indian childhood cirrhosis (ICC) has fascinated the medical fraternity for almost 100 years. The peculiar features, enigmatic etiology and uniformly fatal outcome had frustrated many. In 1978, a finding almost by chance revealed a striking association of exceedingly high hepatic copper content. This finding led to the hypothesis that milk contaminated with copper caused the disease. It was characterized by occurrence of the disease in the infants with dietary history of dietary copper consumption. It is largely disappearing in India because of preventive strategies.

There is a heterogeneous group of patients who do not fit into either WD or ICC. There are case reports of this uncommon disease from other countries also. It was described in Austria , Germany, Saudi Arabia, Kuwait, Japan and USA. In India

largely after the disappearance of ICC it was described as Atypical copper cirrhosis. It is characterized by unexplained liver disease after the 5 years but below 20 years of age with elevated hepatic copper content despite normal copper studies. There are very few reports until now of this peculiar and interesting disease in India. It is still unknown whether it is a milder, adult form of ICC . The causative factors, clinical profile or treatment outcome are still unknown . There was a report of cluster of 138 infants and young children who died from an endemic infantile liver cirrhosis in Western Austria between 1900 and 1974. This disorder, Tyrolean infantile cirrhosis as described by Muller was clinically and pathologically indistinguishable from ICC . It followed autosomal recessive inheritance. Cow's milk fed to the children was contaminated with copper from untinned copper or brass vessels. Muller described it as an Eco genetic disorder which has largely been eradicated through the preventive strategies.

The aim of the present study was to recruit such patients and look into causative factors such as increased dietary copper in the form of history of milk boiled in untinned copper or brass vessels and by measuring drinking water copper levels from birth. The clinical profile and response to copper chelation were also assessed.

Review of literature

WILSON'S DISEASE

Wilson's disease is a rare autosomal recessive genetic disorder of copper metabolism, which is characterised by hepatic and neurological disease. The disease affects between one in 30 000 and one in 100 000 individuals, and was first described as a syndrome by Kinnier Wilson in 1912.[1] Most symptoms first appear in the second and third decades of life. In affected individuals, there is accumulation of excess copper in the liver caused by reduced excretion of copper in bile. The great danger is that Wilson's disease is progressive, can remain undiagnosed, and is fatal if not treated.

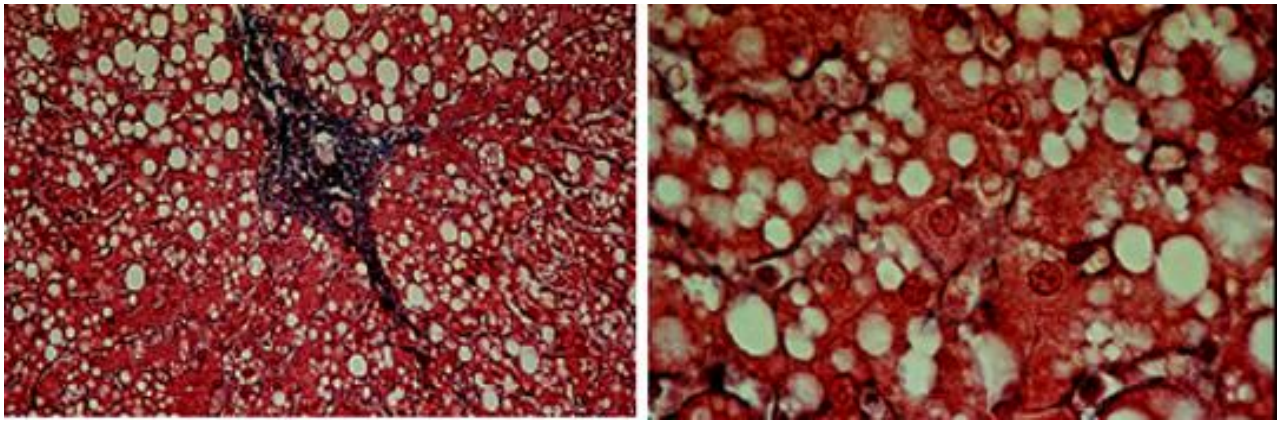
Pathology

In the early stages before cirrhosis develops, histologic findings in the liver consist of steatosis, focal necrosis, glycogenated nuclei in hepatocytes, and sometimes apoptotic bodies. As parenchymal damage progresses, possibly through repeated episodes of lobular necrosis, periportal fibrosis develops. Cirrhosis is usually macronodular but may be micronodular.

Early in the course of WD, hepatocellular copper is bound mainly to metallothionein and distributed diffusely in the cytoplasm of hepatocytes; therefore, results of histochemical stains for copper are negative. As the disease progresses, copper exceeds the capacity of metallothionein and is deposited in lysosomes. These lysosomal aggregates of copper can be detected by special staining techniques for copper or copper-binding protein (such as rubeanic acid or orcein). Copper is usually distributed throughout the hepatic lobule or nodule, but

in the cirrhotic liver, some areas may have no stainable copper at all. If the clinical presentation mimics that of autoimmune hepatitis, liver biopsy specimens reveal classic histologic features of chronic hepatitis, such as interface hepatitis. Inflammation may be severe. Results of Mallory staining for hyalin may be positive, and hepatocellular copper accumulation may be detected. In patients who present with fulminant hepatic failure, liver biopsy confirms preexisting liver disease; cirrhosis may be present; and parenchymal copper is located mainly in Kupffer cells rather than in hepatocytes.

Changes in hepatocellular mitochondria, identified with electron microscopy, are an important feature in Wilson disease.[2] The mitochondria vary in size; the numbers of dense bodies in mitochondria may be increased. The most striking change is dilatation of the tips of the mitochondrial cristae as a result of separation of the inner and outer membranes of the cristae, so that the intercristal space is widened to an irregular cystic shape. The crista resembles a tennis racquet if only the tip is dilated. Involvement of hepatocytes may not be uniform, so abnormalities may be found in some hepatocytes in some lobules and not in others. The mitochondrial changes are probably a consequence of oxidative damage from excessive liver copper.



Left panel: Low power shows portal fibrosis, mild portal inflammation, and fatty infiltration (Masson trichrome).

Right panel: High power view shows fatty infiltration of hepatocytes and two glycogen nuclei (Masson trichrome).

Clinical features

The age at onset of symptoms generally ranges from 6 to about 40 years. Wilson disease with hepatic involvement has been identified in patients younger than 5 years and patients older than 60 years.

Hepatic presentation

Symptoms may be vague and nonspecific, such as fatigue, anorexia, and abdominal pain. Occasionally patients present with a self-limited clinical illness that resembles *acute hepatitis*, with malaise, anorexia, nausea, jaundice, elevated serum aminotransferases, and abnormal coagulation test results. Some patients have a his-tory of self-limited jaundice, apparently caused by unexplained hemolysis.

Patients may present with severe, established *chronic liver disease*—hepatosplenomegaly, ascites, congestive splenomegaly, a low serum albumin level, and persistently abnormal coagulation test results. Some patients have isolated splenomegaly without hepatomegaly. Many of these findings relate more to *portal hypertension* as a consequence of Wilson disease than to the metabolic disorder itself.

WD may manifest in children and young adults as clinical liver disease indistinguishable from *autoimmune hepatitis*.[3] As in autoimmune hepatitis, the onset may be acute. Fatigue, malaise, arthropathy, and rashes may occur; laboratory findings include elevated serum aminotransferase levels, a greatly increased serum immunoglobulin (Ig) G concentration, and detectable nonspecific

autoantibodies such as antinuclear and anti–smooth muscle (anti-actin) antibodies. Wilson disease must be specifically ruled out because the treatment of the two diseases is entirely different. With appropriate treatment, the long-term outlook for patients with Wilson disease that manifests as autoimmune hepatitis appears to be favorable, even if cirrhosis is present.

WD may also manifest as *fulminant hepatic failure*, with severe coagulopathy and encephalopathy.[4] Acute intravascular hemolysis is usually present, and renal failure may develop. Unlike fulminant viral hepatitis, fulminant Wilson disease is usually characterized by disproportionately low serum aminotransferase levels (usually much less than 1500 U/L) at the onset of clinically apparent disease. The serum alkaline phosphatase level is in the normal range or even low for age, and the serum bilirubin level is often disproportionately high as a result of hemolysis.[5] Slit-lamp examination of the eyes may demonstrate Kayser-Fleischer rings . Urinary copper excretion is greatly elevated. Affected patients do not show a good response to chelation treatment and require urgent liver transplantation; albumin dialysis and related techniques may serve as temporary procedures until liver transplantation can be performed.[6]

Recurrent bouts of hemolysis may predispose to the development of *gallstones*. Children with unexplained cholelithiasis, particularly with small bilirubinate stones, should be tested for Wilson disease. Unlike other types of chronic liver disease, Wilson disease is rarely complicated by hepatocellular carcinoma.

In patients who have predominantly hepatic disease, evidence of subtle neurologic involvement often can be found. Mood disturbance (mainly depression, but sometimes impulsive or neurotic behavior), deterioration in school performance or handwriting, and clumsiness may be identified through careful questioning of the

patient or his or her parents. A soft whispery voice (hypophonia) is another early feature of neurologic involvement.

Neurologic presentation

The neurologic presentation of Wilson disease tends to occur in the second and third decades or later but has been reported in children as young as 6 to 10 years. Most patients with a neurologic presentation have hepatic involvement, albeit often asymptomatic. Neurologic involvement follows two main patterns, *movement disorder* and *rigid dystonia*.[7] Movement disorders tend to occur earlier and consist of tremors, poor coordination, and loss of fine motor control. Spastic dystonic disorders generally develop later, with mask-like facies, rigidity, gait disturbance, and pseudobulbar involvement such as dysarthria, drooling, and swallowing difficulty. Intellect is not impaired.

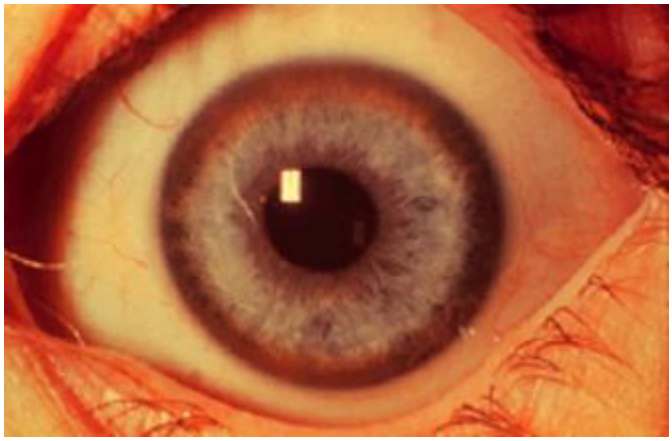
Psychiatric presentation

As many as 20% of patients may present with purely psychiatric symptoms.[8] These symptoms are highly variable, although depression is common. Phobias and compulsive behaviors have been reported; aggressive and antisocial behaviors may also be seen.

Ocular signs

The classic *Kayser-Fleischer ring* is caused by copper deposition in Descemet's membrane of the cornea. Copper is actually distributed throughout the cornea, but fluid streaming favors accumulation near the limbus,

especially at the superior and inferior poles and, eventually, circumferentially around the iris. A careful slit-lamp examination is mandatory.



Kayser-Fleischer ring

Copper deposition in the lens (*sunflower cataract*), which does not interfere with vision, may be seen on slit-lamp examination and, like Kayser-Fleischer rings, disappears with chelation therapy. Kayser-Fleischer rings may be absent in 15% to 50% of patients with exclusively hepatic involvement and in presymptomatic patients, whereas most patients with a neurologic or psychiatric presentation of WD have Kayser-Fleischer rings; only 5% do not. Kayser-Fleischer rings are not specific for WD. They may be found in patients with other types of chronic liver disease, usually with a prominent cholestatic component, such as primary biliary cirrhosis, primary sclerosing cholangitis, auto-immune hepatitis, and familial cholestatic syndromes. Kayser-Fleischer rings have also been reported in patients with nonhepatic diseases.

Involvement of the other organs

WD can be accompanied by various extrahepatic disorders apart from neurologic disease. Episodes of *hemolytic anemia* can result from sudden release of copper into the blood. *Renal disease*, mainly *Fanconi's syndrome*, may be prominent. Findings include microscopic hematuria, aminoaciduria, phosphaturia, and defective acidification of the urine. *Nephrolithiasis* also has been reported. *Arthritis*, affecting mainly the large joints, may occur as a result of synovial copper accumulation. Other musculoskeletal problems are *osteoporosis* and *osteochondritis dissecans*. Vitamin D–resistant *rickets* may develop as a result of the renal damage. Copper deposition in the heart can lead to *cardiomyopathy* or *cardiac arrhythmias*. Sudden death in Wilson disease has been attributed to cardiac involvement but is rare. Copper deposition in skeletal muscle can cause *rhabdomyolysis*. Endocrine disorders can occur. *Hypoparathyroidism* has been attributed to copper deposition. *Amenorrhea* and *testicular problems* appear to result from WD itself, not from cirrhosis. *Infertility* or *repeated spontaneous abortion* may be a sign of WD. *Pancreatitis*, possibly resulting from copper deposition in the pancreas, may also occur.

Diagnosis of Wilson’s disease

AASLD recommendations for diagnosis and screening for Wilson's disease[9]

Clinical features:

WD should be considered in any individual between the ages of 3 and 55 years (Wilson's disease has been diagnosed in patients in their seventies) with liver abnormalities of uncertain cause. Age alone should not be the basis for eliminating a diagnosis of WD.

WD must be excluded in any patient with unexplained liver disease along with neurological or neuropsychiatric disorder.

In a patient in whom WD is suspected, Kayser-Fleischer rings should be sought by slit-lamp examination by a skilled examiner. The absence of Kayser-Fleischer rings does not exclude the diagnosis of WD, even in patients with predominantly neurological disease.

Diagnostic testing:

An extremely low serum ceruloplasmin level (<50 mg/L or <5 mg/dL) should be taken as strong evidence for the diagnosis of WD. Modestly subnormal levels suggest further evaluation is necessary. Serum ceruloplasmin within the normal range does not exclude the diagnosis.

Basal 24-hour urinary excretion of copper should be obtained in all patients in whom the diagnosis of WD is being considered. The amount of copper excreted in the 24-hour period is typically >100 mcg (1.6 micromol) in symptomatic patients, but finding >40 mcg (>0.6 micromol or >600 nmol) may indicate WD and requires further investigation.

Penicillamine challenge studies may be performed for the purpose of obtaining further evidence for the diagnosis of WD in symptomatic children if basal urinary copper excretion is <100 mcg/24 hours (1.6 micromol/24 hours). Values for the penicillamine challenge test of >1600 mcg copper/24 hours (>25 micromol/24 hours) following the administration of 500 mg of D-penicillamine at the beginning and again 12 hours later during the 24-hour urine collection are found in patients with Wilson disease. The predictive value of this test in adults is unknown.
Hepatic parenchymal copper content >250 mcg/g dry weight provides critical diagnostic information and should be obtained in cases where the diagnosis is not straightforward and in younger patients. In untreated patients, normal hepatic copper content (<40-50 mcg/g dry weight) almost always excludes a diagnosis of WD. Further diagnostic testing is indicated for patients with intermediate copper concentrations (70-250 mcg/g dry weight) especially if there is active liver disease or other symptoms of WD.

Neurologic evaluation and radiologic imaging of the brain, preferably by MR imaging, should be considered prior to treatment in all patients with neurologic WD and should be part of the evaluation of any patient presenting with neurological symptoms consistent with WD.

Mutation analysis by whole-gene sequencing is possible and should be performed on individuals in whom the diagnosis is difficult to establish by clinical and biochemical testing. Haplotype analysis or specific testing for known mutations can be used for family screening of first-degree relatives of patients with WD. A clinical geneticist may be required to interpret the results.

Diagnostic considerations in specific target populations:

Patients in the pediatric age bracket who present a clinical picture of autoimmune hepatitis should be investigated for WD.

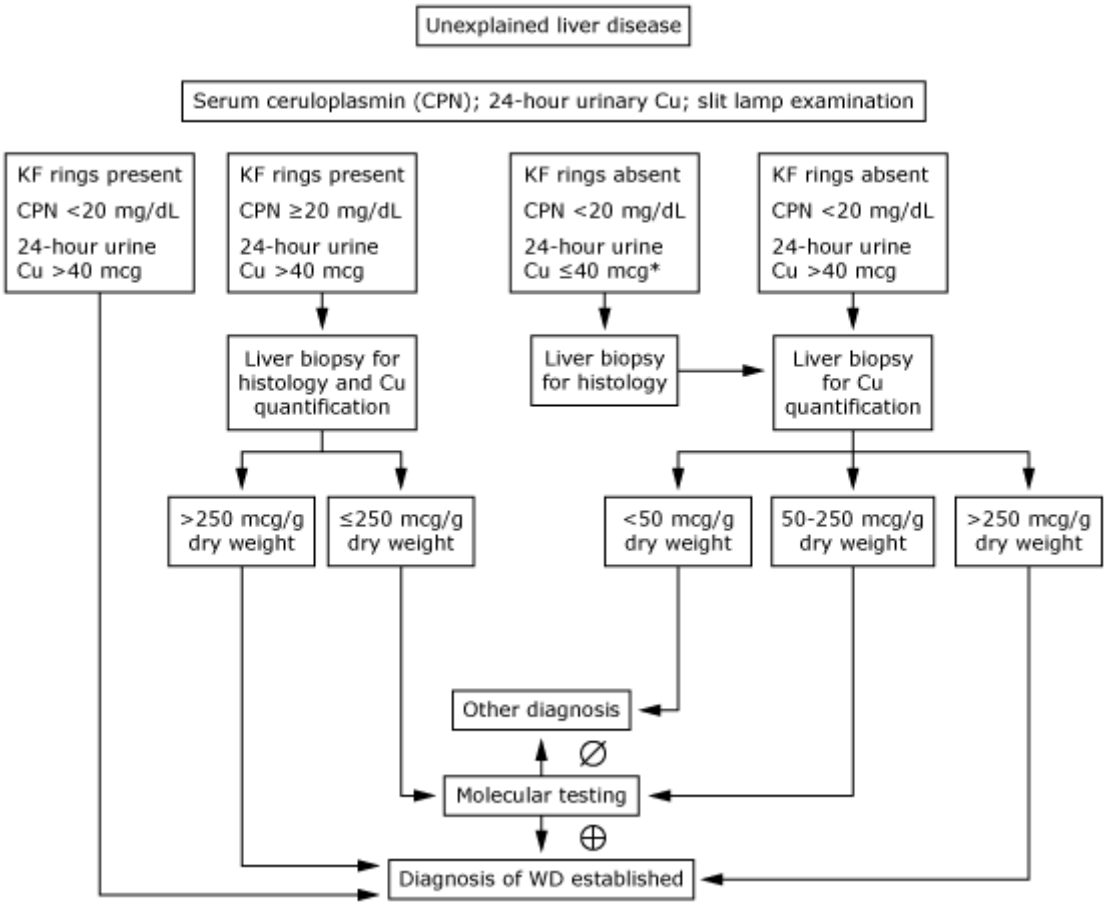
Adult patients with atypical autoimmune hepatitis or who respond poorly to standard corticosteroid therapy should also be investigated for WD.

WD should be considered in the differential diagnosis of patients presenting with nonalcoholic fatty liver disease or have pathologic findings of nonalcoholic steatohepatitis.

WD should be suspected in any patient presenting with acute hepatic failure with Coombs-negative intravascular hemolysis, modest elevations in serum aminotransferases, or low serum alkaline phosphatase and ratio of alkaline phosphatase to bilirubin of <2.

First-degree relatives of any patient newly diagnosed with WD must be screened for WD.

Approach to diagnosis of Wilson disease (WD) in a patient with unexplained liver disease



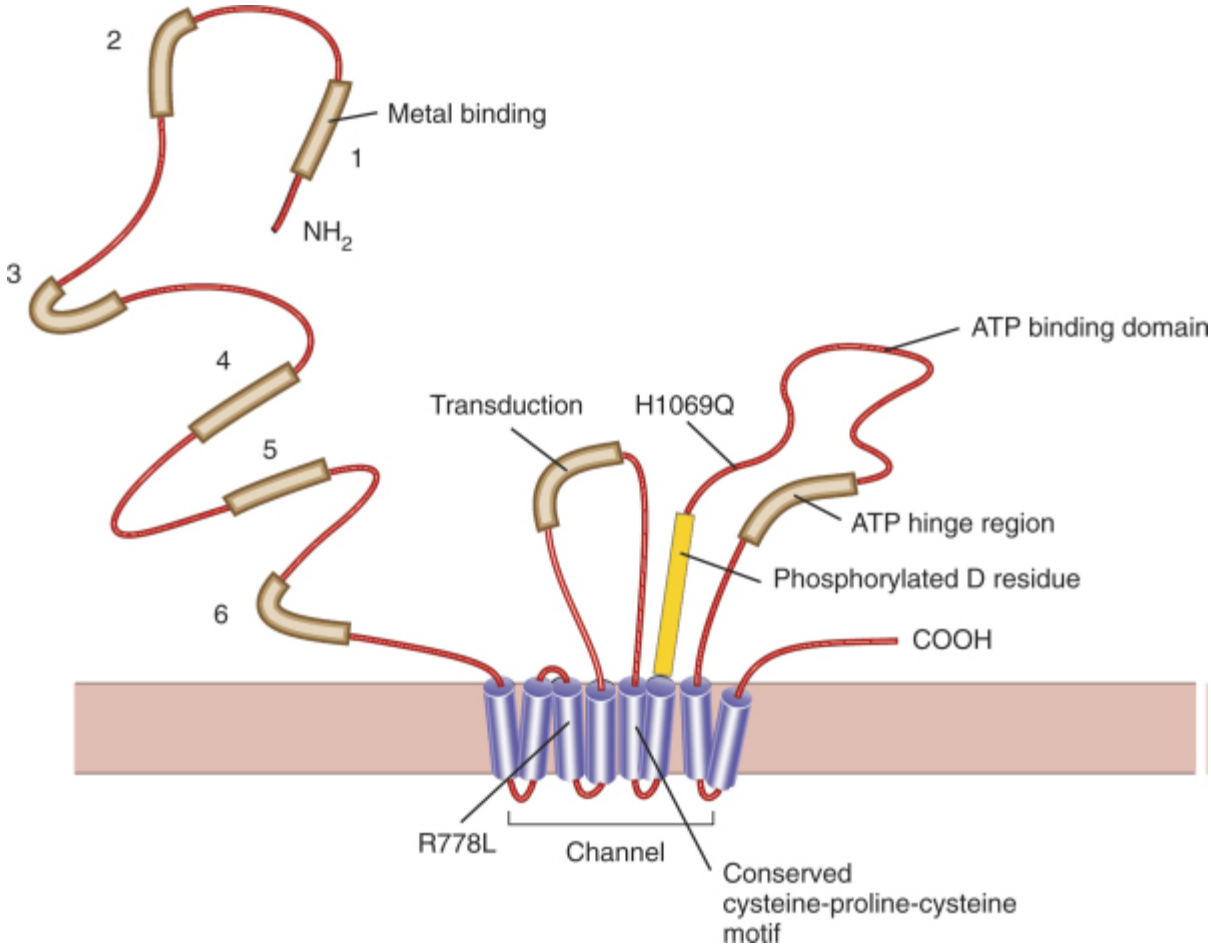
Molecular testing means confirming homozygosity for one mutation or defining two mutations constituting compound heterozygosity.
CPN: ceruloplasmin; KF: Kayser-Fleischer.

Criterion	Abnormality	Advantage	Disadvantage
Kayser–Fleischer corneal rings	Found on slit lamp examination	Easily assessed physical finding	Normal in 10–45% of patients, mainly the young
Serum ceruloplasmin	Less than 20 mg/l	Decreased in 73–95% of patients	Maybe normal, mainly with liver disease
24-hour copper excretion	More than 100 micrograms/ 24 hours	Increased in 85% of patients; useful in acute liver failure	Copper contamination and incomplete sample
Non-ceruloplasmin bound copper	More than 12 micrograms/dl	Increased when ceruloplasmin levels are normal	Not routinely reported
Liver copper quantitation	More than 250 micrograms/g dry weight	Increased in 90% or more of patients	Elevated in chronic cholestasis Sampling errors
Radiocopper scan	Lack of copper binding by ceruloplasmin	Differentiates homozygotes and heterozygotes	Blood samples over 48 hours

Predictive value of diagnostic criteria of Wilsons disease

ATP7B gene

Cloning of the Wilson disease gene (*ATP7B*) was accomplished by a combination of conventional linkage analysis,[10] physical mapping of the relevant region of chromosome 13q14, and recognition of its high homology with the Menkes disease gene. [11,12] The coding region of the Wilson disease gene is 4.1 kilobases in length, with messenger RNA (mRNA) of about 8 kilobases. The product is a membrane P-type adenosine triphosphatase (ATPase) that consists of 1443 amino acid residues and has a molecular mass of 160 kd. There are predicted to be six copper binding domains, a phosphorylation domain, an ATP-binding region, and eight transmembrane domains. All functionally important regions of the gene are conserved between bacteria and yeast. Mutations in the *ATP7B* gene result in retention of copper in the liver as well as impaired incorporation of copper into ceruloplasmin.



A model of the predicted product of the Wilson disease gene, *ATP7B*

Additional proteins are involved in the intracellular transport of copper. Copper is not free in the cell but is transported to specific proteins by copper chaperones.[13] The chaperone ATOX1 transports copper to ATP7B. The study of inherited copper toxicosis in Bedlington terriers has identified a possible new component of the copper transport system. Affected dogs show clinical variability that ranges from death from hepatic disease at 2 to 3 years of age to less severe chronic disease to a very high level of hepatic copper without clinical consequences. The proposed defective canine gene was identified by positional cloning through the use of markers to identify a region containing the *MURR1* gene.[14] This deletion of one exon of the *MURR1* gene is found in most, but not all, affected dogs.[15]

Mutation analysis

More than 260 reported mutations in the *ATP7B* gene have been detected in many different populations by single-strand conformation polymorphism analysis or by sequencing of each of the 21 exons. [16,17,18] These mutations are recorded in the WD Mutation Database.[19] High-throughput methods are making mutation analysis more feasible for this disease, as for many others. Mutation analysis can be carried out with approaches such as denaturing high-performance liquid chromatography[20] or by high-throughput sequencing of either selected or all exons of the gene.

The identification of one mutation may be adequate to confirm the diagnosis of WD, if (1) characteristic clinical symptoms and at least some biochemical features are present and (2) the one mutation detected is clearly established as a disease-causing mutation.

The majority of mutations identified to date in *ATP7B* are missense mutations (57%). Small deletions and insertion (28%), nonsense (7%), and splice site (8%) mutations occur throughout the gene.[19] Various ethnic groups have different specific mutations. The common histidine1069glutamine (His1069Gln) mutation[21] is present at least in the heterozygous state in 35% to 75% of Europeans with Wilson disease; the higher rate is relevant only for Eastern Europe.[22] Exon 8 of the gene is particularly rich in mutations in European populations; depending on the age of onset, at least one mutation can be identified in exon 8 in about 50% to 60% of patients.[23] The mutation arginine778leucine is common in Chinese populations.[24] Because no mutation is present in high frequency in Japanese and Mediterranean populations, mutation detection is more challenging in such populations. In some populations that have ethnic homogeneity or in which the spectrum of mutations is established, testing strategies can identify the mutations

in more than 90% of patients, as in Sardinians, among whom Wilson disease occurs with a frequency of 1 in 7000 live births.[25] If the patient is clinically normal, has only slight signs of the disease, or has a late age of onset, that patient could actually be a heterozygote. However, up to now, heterozygotes have not been known to become clinically affected or to require treatment.

No individual biochemical test is reliable for the identification of patients. In some cases, even all combinations of tests prove inadequate for a diagnosis. The use of molecular tests in patients with any clinical symptoms of the disease may become routine in the near future and is already feasible in some populations.

Mutation analysis – Indian perspective

The spectrum of ATP 7 B mutations in patients with WD, mostly from three southern Indian states and their family members were studied. The spectrum of ATP 7 B mutations were described including 11 novel mutations in Indian WD patients and were documented lack of single dominant mutation. Identical WD phenotype among siblings in 6 of 8 families with > 1 child affected by WD suggests that factors other than ATP 7 B mutation influence WD phenotype.[26]

In eastern India , WD patients from 109 unrelated families and their first-degree relatives comprising 400 individuals were enrolled to study the spectrum of ATP 7B mutations. In addition to previous reports, eight mutations including three novel (c.3412 + 1G > A, c.1771 G > A, c.3091 A > G) variants were identified. 17 mutations were identified in eastern India including five common mutations that account for 44% of patients. Comparative study on WD mutations between different regions of India suggests high genetic heterogeneity and the absence of a single or a limited number of common founder mutations. Genotype–phenotype

correlation revealed that no particular phenotype could be assigned to a particular mutation and even same set of mutations in different patients showed different phenotypes.[27]

In north west states of India there was absence of common mutations H1069Q, R778W and R778L mutations in WD patients. R778W and I1102T mutations were present in 36% of WD patients.[28] The copper ATPase activity in WD patients was significantly reduced (50%) than that of control individuals. No significant difference was observed in copper stimulated ATPase activity between homozygous (R778W/R778W, I1102T/I1102T) and compound heterozygous (R778W/unknown mutation, I1102T/unknown mutation) WD patients.[28]

Treatment of Wilsons disease

AASLD recommendations for treatment of Wilson's disease (WD)

Initial treatment for symptomatic patients should include a chelating agent (D-penicillamine or trientine). Trientine may be better tolerated.

Patients should avoid intake of foods and water with high concentrations of copper, especially during the first year of treatment.

Treatment of presymptomatic patients or those on maintenance therapy can be accomplished with a chelating agent or with zinc. Trientine may be better tolerated.

Patients with acute liver failure due to WD should be referred for and treated with liver transplantation immediately.

Patients with decompensated cirrhosis unresponsive to chelation treatment should be evaluated promptly for liver transplantation.

Treatment for WD should be continued during pregnancy, but dosage reduction is advisable for D-penicillamine and trientine.

Treatment is lifelong and should not be discontinued, unless a liver transplant has been performed.

For routine monitoring, serum copper and ceruloplasmin, liver biochemistries and international normalized ratio, complete blood count and urinalysis (especially for those on chelation therapy), and physical examination should be performed regularly, at least twice annually. Patients receiving chelation therapy require a complete blood count and urinalysis regularly, no matter how long they have been on treatment.

The 24-hour urinary excretion of copper while on medication should be measured yearly, or more frequently if there are questions on compliance or if dosage of medications is adjusted. The estimated serum non-ceruloplasmin bound copper may be elevated in situations of nonadherence and extremely low in situations of overtreatment.[29]

Penicillamine

D-Penicillamine contains a free sulfhydryl group that functions as a copper chelating moiety. Its major effect is to promote urinary excretion of copper, although it may also function by other mechanisms. Penicillamine reduces the affinity of proteins for copper, thereby allowing previously protein-bound copper to bind to penicillamine. It has also been used in the treatment of rheumatoid arthritis and cystinuria.

Dosing — The drug should be introduced with incremental doses of 250 to 500 mg/day increased by 250 mg increments every four to seven days to a maximum of 1000 to 1500 mg daily in two to four divided doses. This regimen may reduce the incidence of early adverse side effects such as fever and rash, but does not appear to reduce the incidence of late-onset toxicity such as the nephrotic syndrome .A lower dose (750 to 1000 mg daily in two divided doses) is sufficient during the maintenance phase (usually after four to six months). The therapeutic response to changes in maintenance dosage usually will not become evident for six to eight weeks. The dose in children is 20 mg/kg per day (rounded to the nearest 250 mg) given in two or three divided doses.

Penicillamine should ideally be given one hour before or two hours after meals since food interferes with its absorption. However, some patients may require dosing closer to food intake, which is an acceptable compromise if it increases compliance.

Adverse effects

Penicillamine is associated with multiple side-effects leading up to about 5 percent of patients to discontinue therapy. Other treatments should probably be used in patients who are at increased risk for toxicity including those with a history of renal disease, severe thrombocytopenia, or an autoimmune tendency.

Crossreactivity to penicillin may occur. Thus the drug should be used cautiously in patients with known penicillin allergy.

Early sensitivity reactions (occurring within one to three weeks of beginning therapy) are characterized by fever, cutaneous eruptions, lymphadenopathy, neutropenia, thrombocytopenia, and proteinuria. The drug should be discontinued immediately in such patients and alternative treatment (principally trientine) begun.

Several side effects occurring months to years after initiating therapy have also been described. The appearance of proteinuria may herald the onset of nephrotic syndrome, which may occur years after treatment begins. The onset of nephrotic syndrome necessitates cessation of the drug. However, protein excretion of up to 2 g/day produces no symptoms and may not progress. Although cessation of therapy always leads to resolution of the proteinuria, the mean time required for this to occur is approximately one year and some patients take more than two years [30]. A much less common but more serious renal complication is the development of a crescentic glomerulonephritis.

Other later reactions include Goodpasture syndrome, bone marrow toxicity including severe thrombocytopenia or total aplasia, myasthenia gravis, polymyositis, hepatotoxicity, loss of taste, and a lupus-like syndrome characterized by hematuria, proteinuria, a positive antinuclear antibody.

Skin changes have been described including elastosis perforans serpiginosa pemphigus, lichen planus, and aphthous stomatitis [31]

Nausea, vomiting, and anorexia are dose-related signs of gastric irritation and improve if the dose is reduced.

Aplastic anemia is rare but may not reverse with the cessation of therapy.

The neurologic status of patients with predominantly neurologic symptoms worsens in approximately 10 percent of patients after beginning treatment [32]. Furthermore, new neurologic symptoms may appear in some patients, although they are usually transient as long as chelation therapy is continued. These events may be caused by two potential mechanisms: mobilization of hepatic copper stores leading to increased brain copper exposure, or the development of intracellular copper complexes [33].

Penicillamine inactivates pyridoxine. Thus, small doses of pyridoxine, 25 mg per day, should be given to patients treated with penicillamine to prevent pyridoxal phosphate deficiency.

Oral zinc — Oral zinc interferes with the absorption of copper, providing a rationale for its use in Wilson's disease. Zinc induces metallothionein (an endogenous chelator of metals) in enterocytes, which has a greater affinity for copper than for zinc, causing it to bind luminal copper and thereby preventing its entry into the circulation [34]. The bound copper is excreted fecally during normal turnover of enterocytes. Zinc may also induce hepatic metallothionein [35].

Dosing — There are several forms of oral zinc salts, which probably have similar abilities to interfere with copper absorption, but differ in their tolerability. Zinc acetate (Galzin, GATE Pharmaceuticals) causes the least gastrointestinal side-effects while gluconate is more tolerable than sulfate. Dosing is in milligrams of elemental zinc. The dose of zinc acetate in adults and older children is a total of 150 mg daily given in three divided doses. Smaller children (less than 50 kg body weight) should be given a total of 75 mg daily in three divided doses [36]. The optimal dose for children younger than five is unknown. Dosing of zinc should be separated from food and beverages by at least one hour.

Adverse effects — The most common adverse effect of zinc is gastrointestinal upset, appears to be least with zinc acetate. Hepatic deterioration (including a

fatality) has been described in case reports [37]. Neurologic deterioration is uncommon. Elevation in serum amylase and lipase without clinical evidence of pancreatitis has been observed.

Other drugs, such as Trientene and tetrathiomolybdate are recommended that are mainly useful when there are neurological symptomps which are aggravated by pencillamine. Potassium sulfide and carbacrylamine resins, which bind copper in the gastrointestinal tract, are not recommended. Dimercaprol (BAL), the first drug that successfully treated Wilson's disease, is rarely used.

Indian childhood cirrhosis

In October 1887, Boyle Chunder Sen[38] presented the first clinical account of this condition, attributing it to some 'inherited dyscrasia'. In the very next year, Gibbons performed the first autopsy, soon followed by three more. He designated the disease 'Infantile Biliary Cirrhosis', due to the presence of proliferated bile ducts, and ascribed it to some 'endogenous chemical irritant'[39] which went unchallenged for over six decades. The detailed autopsy studies revealed several new and unique features[40] like 'Peri-cellular fibrosis' in the liver, and raised suspicions of 'congenital syphilis', but was ruled out on the basis of 'negative serology'. Since cirrhosis was also accompanied by 'phlebosclerosis' of some of the tributaries of the hepatic vein, the causative role of some exogenous 'plant toxins' was invoked and a new term'Sub-acute toxic cirrhosis' was introduced[40]. However in 1954, Bhende and Deoras described the presence of 'hyaline' in autopsy liver samples and also designated the condition as 'Infantile cirrhosis'[41]. Towards the end of 1970s, Tanner and associates modified the previous histochemical studies on ICC[42], and reported an increase of Orcein +ve stainable hepatic Copper binding protein (CuBP)[43]. Finally in 1979, a new theory of 'dietary copper toxicity', based on elevated levels of hepatic copper, attributed to use of 'copper yielding utensils' was proposed[44]. Soon, the need to confirm the qualitative histochemical studies by 'quantitative elemental analyses' was felt and so the standard Atomic Absorption Spectrometry (AAS) Chemical Analysis was carried out on liver biopsy samples[44]. Almost simultaneously, Popper with the help of Delhi group carried out 'AAS studies on three autopsy samples of ICC[45].

Clinical and epidemiologic features of ICC

The characteristic clinical and epidemiologic features of ICC are as follows[46,47,48,49]

1. The specific age range is from 6 months to 5yrs with a mean of 18 months

2. The disease is predominant in males.

3. There are high rates of parental consanguinity in families affected with this disease, and up to 22% of siblings are affected.

4. The disease is restricted to the Indian subcontinent, and the origin of cases is rural rather than urban.

5. Distribution by religion and caste reflects the local rural population,but Muslims and Christians are mostly spared.

6. The onset is mainly insidious (86%) with nonspecific complaints such as abdominal distention, irregular fever, excessive crying, and altered appetite. In a few children, the disease begins with jaundice, but this is usually a late feature. The feel of the liver is characteristically firm to hard with a sharp, “leafy” edge. Untreated, the progress is relentless and within a few months the affected child is desperately ill with hepatosplenomegaly, ascites, edema, and jaundice. An unusual late feature is an enlarged, palpable gall bladder, believed to be due to cholangitis and cholecystitis. Death is usually due to bleeding, secondary infection, or hepatic coma.

7. Standard liver function tests are usually abnormal but not diagnostic. Clinical or biochemical differentiation of early ICC from other childhood liver disorders such as unresolved viral hepatitis, chronic persistent or active hepatitis, veno-occlusive disease, and cryptogenic cirrhosis is difficult and hence histopathology remains the cornerstone of definitive diagnosis.

Histopathology

The striking orcein-rhodanine staining representing copper is seen consistently and easily in a simplified histologic diagnosis of ICC[50]. The two most discriminatory features of ICC now recognized are widespread,coarse, dark-brown orcein staining and intralobular pericellular fibrosis [51]. Hepatocytic necrosis (seen in 97% of cases) and hyaline (seen in 66% of cases) are also diagnostic though late features. Portal fibrosis, inflammation, and disruption of the limiting plate are seen in most cases, but are seen also in other liver disorders and hence are not of discriminatory value. Parenchymal fat is usually absent and cholestasis is a late feature[52].

Pathogenesis

The increased copper in ICC may be secondary to liver damage and impaired biliary copper excretion [53,54] may be the result of an inherited disorder of copper metabolism akin to Wilson disease or may simply be the result of excessive copper intake. No studies to date have really substantiated the first two suggestions in typical ICC in India. However, many epidemiologic studies and feeding histories strongly support the copper-ingestion theory [55,56,57]. Copper and brass (an alloy of 70% copper and 30% zinc) vessels have been used extensively in India, especially

by traditional Hindu families. Experimentally, boiling and storing milk in untinned brass vessels raises its copper concentration more than 60 times-a gross copper contamination. Water takes up copper less avidly-storing water in a copper vessel raises its modest copper concentration only about six times[55] . Ultracentrifugation and chromatography studies have shown that copper binds predominantly to casein, which is an exclusive constituent of milk. In acidic pH (as in gastric juice) casein liberates most of this bound copper, making it available for rapid absorption [58]. Boiling or storing milk or water in stainless steel or aluminum does not change the copper concentration. Traditional "kaIhai" or "tinning" of copper and brass vessels prevents copper contamination, yet this procedure is often neglected because of cost and effort.

Prevention of Indian childhood cirrhosis

The copper-ingestion hypothesis of ICC suggested the remarkable possibility that a fatal liver disorder could be eradicated through a simple message of health education. This was demonstrated in Pune through an extensive interventional study [59]. A massive campaign of health education involving government and nongovernment health agencies (district health officials, multipurpose workers, Anganwadi workers, dairy societies, and adult education workers) was carried out in the entire Pune district from 1984 to 1987. Systematic surveys showed that the campaign reduced the use of brass vessels in the Pune district from 13% to 4%. This was associated with a significant fall in the number of cases of ICC from the Pune District seen at KEM Hospital. Dairy development and government milk schemes have led to replacement of the milkman's brass vessels with aluminum canisters and glass or plastic bottles for milk supply. With urbanization and smaller

families now the norm, household brass vessels have been put away in favor of cheaper and hardier stainless steel or aluminum containers.

Treatment of Indian childhood cirrhosis

Therapeutic trials of the copper chelator D-penicillamine for ICC treatment have shown a remission in up to 65% of patients in the early (preicteric) stage of disease [60]. Remission is associated with clinical recovery, reduction in hepatic copper to normal concentrations, and striking histologic reversal of cirrhosis within a couple of years of therapy [61,62,63]. Twenty-nine such survivors of ICC (followed up for 5-12 y) have continued to do well despite the withdrawal of D-penicillamine after 3-5years of treatment The continued well-being of ICC survivors without Dpenicillamine and the disappearance of ICC coincident with a decrease in the use of brass vessels for feeding strengthen the evidence that copper accumulation in ICC is an acquired phenomenon , rather than an inborn error of copper metabolism.

Characteristics of Wilson disease and Indian childhood cirrhosis (ICC): two pediatric copper storage disorders

	Wilson disease	ICC
Age	> 5 y	6 mo to 5 y
Family history	Autosomal recessive	Yes, in siblings
Presentation	Extremely variable	Characteristic
Hepatic copper (μg/g)	Raised (> 800)	Grossly raised (> 1000)
Ceruloplasmin (mg/L)	Low	Normal or high
Histology	Nuclear glycogen, chronic active hepatitis (+/−), cirrhosis (+/−)	Pericellular fibroses, hepatocytic necrosis, hyaline
Orcein staining	+/−	+++
Etiology	Deranged copper metabolism	Copper ingestion: too much, too soon
D-Penicillamine	Necessarily lifelong	Can be withdrawn on recovery

Recent views about ICC[64]

The newly invoked theory of ‘dietary copper toxicity’ was found to be virtually untenable. Thus, in four of the six Centres, use of copper yielding utensils was reported in just 10 to 50 per cent of cases categorized as definitive ICC, a frequency not different from that in controls. In the remaining two Centres located in Mumbai, copper yielding utensils were not at all used for cooking and boiling or storing milk for any of the definitive ICC cases. Until they developed the disease, 9 children with definitive or florid ICC were found to be purely breast-fed without any supplementation by milk or weaning diets. All the other stages of ICC were also encountered in the total absence of use of copper yielding utensils for cooking the food of affected children.

The causative role of Cu has been dispelled. In view of the subsequent disappearance of the disease in the affected castes , the hypothesis of inherited susceptibility of copper metabolism also does not appear to be valid. Instead, the possible hepato-toxic effects of post-puerperal domestic therapeutic remedies appear to be more plausible. The current efforts at identification of the incriminated compound(s) in specific herbal formulations, by appropriate animal experiment, will resolve the mystery of ICC.

Atypical copper cirrhosis

There is a heterogenous condition which neither fits into WD nor ICC described as Atypical copper cirrhosis.(ACC)

In 1995 Bhanumathy Ramakrishna et al[65] working on liver biopsies in children with cryptogenic cirrhosis found that they have copper deposition in the liver with out either WD or ICC , as defined according to the accepted clinicopathological findings in these conditions. The children with ACC presented at a mean age of 9.8 years with abdominal distension and organomegaly with or with out ascites or jaundice. None had Kayser Fleisher rings. Serum and urine copper levels were elevated only in 2 of 7 children studied. Serum ceruloplasmin levels were normal or increased . No history of possible excess copper ingestion was obtained except in the case of one child whose family used brass vessels to store drinking water.

Pathology

Tissue from patients with ACC showed micronodular cirrhosis . Fatty change was absent from all the biopsy samples. Varying degrees of pericellular fibrosis, ballooning of hepatocytes and Mallory's hyaline were seen. Stainable copper or copper associated protein was present in all the biopsies.

All patients received copper chelation with improvement of symptoms.

ICC like cirrhosis

Recent reports of ICC-like cirrhosis with raised hepatic copper concentrations in Western countries have raised doubts yet again about the copper-ingestion theory of ICC [66,67,68,69]. Although copper-contaminated water (copper pipes and water with a low pH) has been incriminated in a few cases, the amount of copper ingested does not match that in ICC in India.

ICC like illness was seen in Austria[70] , Kuwait[71], Saudi Arabia[72] , Japan[73] and Germany[74].

Endemic infantile Tyrolean cirrhosis[70]

138 infants and young children died from an endemic infantile liver cirrhosis in a circumscribed rural area of western Austria between 1900 and 1974. Frequency of the disease peaked between 1930 and 1960. It has disappeared from this area since 1974. Clinical and genetic data on the patients was gathered; pedigrees analysed and ethnographic studies and interviews were undertaken. The disease, which was clinically and pathologically indistinguishable from Indian childhood cirrhosis and hepatic copper toxicosis, was transmitted by autosomal recessive inheritance. Cow's milk, contaminated with copper from untinned copper or brass vessels, may have contributed to the development of copper toxicosis. Replacement of untinned copper cooking utensils by modern industrial vessels has eradicated the disease.

The findings strongly suggest that the endemic Tyrolean childhood cirrhosis-and by analogy non-Wilsonian hepatic copper toxicosis occurring elsewhere-is an ecogenetic disorder requiring the involvement of both genetic and environmental factors for the disease to become manifest.

Aims of the study

1. To look for evidence of increased copper intake in Atypical copper related liver disease (ACLD) patients.

2. To study clinical features and treatment outcome of ACLD patients.

Materials and methods

This study was conducted in Department of Clinical gastroenterology and hepatology, Christian medical college , Vellore. It was approved by institutional research and ethics committee (Institutional review board).

Study design :

Prospective descriptive study

Time period :

From Dec 2006 to Dec 2008

Inclusion criteria :

Patients with chronic liver disease with significant stainable copper deposits[83] aged less than 20 yrs and more than 5 years

Exclusion criteria:

Wilson's disease
Indian Childhood Cirrhosis
Biliary diseases
Positive viral markers
Active alcohol intake
Age more than 20 yrs

Subjects:

Patients presenting to outpatient and / or inpatient department of Clinical gastroenterology and hepatology , Christian medical college , Vellore from Dec 2006 to Dec 2008 with the confirmed diagnosis of Atypical copper related liver disease were included in the study after informed consent.

All patients were subjected to etiological evaluation including liver biopsy. Patients with Wilsons disease (leipzig criteria), Indian childhood cirrhosis and cholestatic disorders were excluded from the study

Dietary history was obtained in all the subjects. Whether drinking water was stored in brass vessels (tinned or untinned) or cooking was done using brass vessels (tinned or untinned) was obtained in all the subjects.

Drinking water was obtained from different regions of residence right from the birth of the study patients. Drinking water was tested for copper content using flame atomic absorption spectrometry

Diagnostic criteria of Atypical copper related liver disease

Atypical copper related liver disease was defined as unexplained liver disease

a) In 5 - 20 years old patients

b) Significant copper deposits on liver biopsy[83] (by Rhodanine/Orcein stain)

c) Normal or high serum ceruloplasmin,

d) No Kayser Fleischer ring,

e) Absent ATP7B mutation

f) Histological features not consistent with WD or ICC on liver biopsy, and

g) No history of copper ingestion (cooking in brass vessels).

11 patients were included in the study who satisfied the above criteria.

Drinking water sample Collection Procedures[75]

Samples were collected from water taps that were undisturbed for at least six hours, but no more than 12 hours. Therefore, patients were asked to take samples first thing in the morning. This minimum six-hour standing time helps to standardize the test results. Copper levels increase as long as water stands in a home's plumbing system. Water that stands longer than 12 hours may have

high copper levels that do not represent typical conditions. The action level is 1.3 mg/L for copper.

Standardized containers for specimen collection were provided to the patients from our institution.

Measurement of serum ceruloplasmin

The methods used to measure ceruloplasmin were o-dianisidine dihydrochloride method [82] before 1st Nov 2007 (normal range , 62 – 140U/L) and ferroxidase method[76] on and after 1st Nov 2007(normal range , 200 – 1100 U/L) .

O-DIANISIDINE DIHYDROCHLORIDE METHOD[82] :

Principle of the test :

O-dianisidine dihydrochloride (4,4'-diamino-3,3'-dimethoxy -biphenyl) is used as a substrate. This reagent which requires no purification of the commercially available material and is stable in aqueous solution, is converted into a yellowish-brown (16) reaction product in the presence of ceruloplasmin and oxygen at pH 5. Acidification stops the enzymatic reaction, and a stable purplish- red (16) solution is formed that absorbs maximally at 540 nm. This is read using spectrophotometer.

Procedure

Pipet 0.75 ml of acetate buffer and 0.05 ml of serum into two tubes (one marked “5 min,” and the other “15 min”). Place the tubes in a 30^0 C water bath. Allow 5min for temperature equilibration before pipetting, at timed intervals, 0.2 ml of *o*-dianisidine dihydrochloride reagent (pre-incubated at 30^0 C) into each tube, starting the timer at the first substrate addition. After exactly 5 min remove the “5 min” tube from the water bath, add 2.0 ml of the 9 molar sulfuric acid, and mix immediately. At exactly 15 min remove the “15 min” tube, add 2.0 ml of the 9 molar sulfuric acid and mix immediately. Measure the absorbance of the purplish-red solutions at 540nm, in a cuvet having a 1-cm light path vs. de-ionized water as a blank.

FERRO OXIDASE METHOD[76] :

Analyzer : Hitachi 912

Summary & explanation of the test

Functional ceruloplasmin or copper oxidase, is measured colorimetrically using the reaction of ferene with ferrous ions remaining after the ferroxidase of ceruloplasmin The method used is a fixed time kinetic, with a 2 point inverse calibration.

Principle of the method

The chromogen is 3-(pyridyl)-5,6-bis (2-(5-furyl sulfonic acid)) – 1,2,4-tri – azine , disodium salt. The serum sample is incubated with a known amount of ferrous ions, which are oxidized to ferric ions by the enzyme. The chromagen forms a highly colored complex with ferrous ions , but not with ferric ions.

At the end of the incubation period , the chromgen is added and the remaining non oxidized ferrous ions measured photometrically. The difference in the ferrous ion concentration before and after the enzymatic reaction indicates the amount of oxidized ferrous ion.

Measurement of 24 hr urinary copper[77]

Principle of the method

When a diluted serum sample is aspirated into an atomic absorption spectrophotometer , " the ground state " atom absorbs light energy of a specific wavelength as it enters the excited state. As the number of atoms in the light path increases , the amount of light absorbed also increases. By measuring the amount of light absorbed also increases. By measuring the amount of light absorbed , a quantitative determination of copper in the sample could be made.

Sample preparation

A 1:2 diluted urine (1 ml urine + 1ml of deionised water) is directly aspirated and the reading obtained is then compared with a copper standard containing 2mg/L for serum copper and 200 ug/L for urine copper respectively. Multiply the value obtained x2 to get the final value.

Measurement of drinking water copper level[84]

This method is based on the cloud-point extraction (CPE) technique for the trace analysis of Cu in water samples .The analytes in the initial aqueous solution are complexed with pyrogallol, and 0.1%(w/v) Triton X-114 is added as surfactant. Following phase separation at 50^{o}C, based on the cloud point of the mixture and dilution of the surfactant-rich phase with acidified methanolic solution, the enriched analytes are determined by flame atomic absorption spectrometry.

Kayser Fleisher (KF) ring examination :

KF ring was examined by slit lamp examination by ophthalmologists in Department of Ophthalmology , Schell eye hospital, Vellore.

Liver biopsy :

All ACLD patients were subjected to liver biopsy either via percutaneous route or transjugular route depending on the presence of coagulopathy and / or ascites. The biopsy specimen was sent for histopathology and dry weight liver for copper estimation .

The presence of stainable copper and copper associated protein (CAP) was graded semi quantitatively[83] as follows

0 - negative;

1+ - present in a few hepatocytes;

2+ - present in several hepatocytes;

3+ - abundant copper and CAP in most hepatocytes, usually visible on naked eye examination of the slide.

If there is more than or equal to 2+ copper deposition on liver biopsy it was taken as significant copper deposition.

Estimation of dry weight liver copper : [86]

Principle of the test :

The test is based on measuring copper from 15-mg (wet weight) sample of human liver by atomic absorption spectrophotometry. The sample is digested with nitric acid (1.0 mol/liter), evaporated, and dilute HNO3 (10 mmol/Iiter) added. The reconstituted acid mixture is injected into the graphite tube atomizer for analysis of Cu .

Tissue Processing :

Portions of human liver tissue, normal on gross examination were frozen at -20^{o}C until needed for analysis. Adjacent pieces were histologically examined. The portions of liver frozen were complete cross sections, about 3 cm thick, surrounded completely by capsule. Before digestion, the liver was thawed and the capsule stripped away, revealing a clean, uncontaminated surface. The sample was placed in a dried, preweighed, acid-cleaned, 3-ml test tube and the wet weight of the tissue was then recorded. After this weighing, the tubes were placed in a beaker on a hot plate, and the beaker was covered with an inverted larger beaker. The air temperature inside the larger beaker was 80^{o}C. tissue was dried for 24 h to constant weight, cooled in a desiccator,and weighed. About 70% of the wet weight

was lost, leaving about 5.0 mg of dry liver tissue. One milliliter of 1.0 mol/liter HNO3 acid was then added, and the dried sample was acid digested on a hot plate at 80^{o}C another 24 h. The 1.0 mol/liter HNO3 was then slowly evaporated on a hot plate and 2.0 ml of 10 mmol/liter HNO3 was added with vigorous mixing. The liver tissue almost always completely dissolved. Incomplete solubilization did not alter the analytical results. Analysis was delayed for 4-6 h after reconstitution. The concentration of the three metals in the tissue was such that only 30 ul of the reconstituted solution of liver tissue was used for Cu analysis.

A Model 303 atomic absorption spectrophotometer and a graphite tube atomizer was used for all Cu analyses.

ATP 7B mutation analysis – assay procedure

Overview of the test :

DNA was isolated from peripheral blood leukocytes of the patients after obtaining informed consent. DNA samples were electrophoresed on Agarose gel to confirm DNA isolation. All the exons (21 exons of ATP7B gene) were amplified by Polymerase Chain Reaction PCR product heteroduplexed with control DNA sample. Conformation Sensitive Gel Electrophoresis was done for heteroduplexed sample.

Conformation Sensitive Gel Electrophoresis (CSGE) (mutation screening)

The 21 exonic and flanking intronic regions of ATP7B gene were amplified by polymerase chain reaction using described primers[78,79] . Each 25µl reaction contained 12.5µl of 2X ready reaction mix and the concentration of the primers used for each reaction and the annealing temperatures are shown in Table 1. Following an initial denaturation at 95^{o}C for 5 minutes, 35 cycles of PCR amplification at 94^{o}C for 40 seconds, a 40 seconds annealing at the temperature shown in Table 1 and extension at 72^{o}C for 40 seconds were performed.

To our knowledge, this is the first study reporting the use of CSGE to screen for ATP7B mutations in Atypical copper related liver disease subjects[80] . Briefly, 3 µl of PCR product from each patient was mixed with an equal volume of PCR product from a sequenced normal control correlating with the gene bank. Samples were denatured at 95^{o}C for 5 minutes and then incubated at 55^{o}C for 45 minutes to allow heteroduplex formation. 3 µl of heteroduplexed samples were mixed with 2 µl of loading buffer and loaded onto a 10% CSGE gel. To attain the equilibration of the buffer throughout the gel 1hour pre-run was given with 750 V/cm. Following electrophoresis (450 V/cm, 17 hours), bands were visualized by ethidium bromide staining and samples displaying abnormal CSGE profiles relative to that in a normal individual were sequenced using ABI310 genetic analyzer.

Table 1: Amplimers and Annealing Temperature for CSGE analysis and sequencing

Exon	Amplimers	Annealing Temperature
1	5'TTCCCGGACCCCTGTTTGCT3' 5'AATCCTCCTGGTGGGAGTGAG3'	56C
2a	5'GTTTCAAGGTTAAAAAATGT3' 5'GGCACATATTTCACAGTGG3'	45C
2b	5'GGCCACCAGCACAGTC3' 5'CTGGGCAGGCAAGGAC3'	59C
2c	5'GAGGCCAGCATTGCAGA3' 5'AGCCACTTTGCTCTTGATG3'	50C
2d	5'ATGACATGGGATTTGAAG3' 5'TCCGACAGGAAGAGAAAC3'	48C
2e	5'GCCCAAGTAAAGTATGACCC3' 5'GACACCGATATTTGCTGCAC3'	50C

2f	5’AGGGCTACCTATACACCATCC3’	50C
	5’AGGGCTACCTATACACCATCC3’	
3	5’GATATTTCTGACATTTTATCC3’	45C
	5’GCAGCATTCCTAAGTTCA3’	
4	5’CCACCCAGAGTGTTACAGCC3’	52C
	5’ACCCCCTAACGCACCCA3’	
5	5’CCTGGGTCTGTGGGATTCT3’	50C
	5’AAAGGTGACTACAATTTTTAATGA3’	
6	5’CTGCCAATGCATATTTTAAC3’	50C
	5’GGTAGAGGAAGGGACTTAGA3’	
7	5’TGTAATCCAGGTGACAAGCAG3’	52C
	5’CACAGCATGGAAGGGAGAG3’	
8	5’AACCCTTCACTGTCCTTGTC3’	48C
	5’AGGCAGCTCTTTTCTGAAC3’	
9	5’TTTCGATAGCTCTCATTTCACA3’	50C
	5’TGCCCACACTCACAAGGTC3’	
10	5’AGTCGCCATGTAAGTGATAA3’	48C
	5’CTGAGGGAACATGAAACAA3’	
11	5’CTGTCAGGTCACATAGTGCT3’	50C
	5’TTTCCCAGAACTCTTCACA3’	
12	5’CTTGTGTGTTTTATTTCTTC3’	48C
	5’ACCACCATATAGCCCAAG3’	
13	5’TGAACTCTCAACCTGCCT3’	50C
	5’TCTCAGATGGGAAAGCCG3’	
14	5’TCCATCTGTATTGTGGTCAG3’	50C
	5’CAGCTAGGAGAGAAGGACAT3’	
15	5’CTTTCACTTCACCCCTCT3’	48C
	5’AGCTGACAGAGACAAAAGC3’	
16	5’CCATTTAGAAATAACCACAG3’	48C
	5’AGGAAGGCAGAAGCAGA3’	
17	5’CAAGTGTGGTATCTTGGTG3’	50C
	5’CTGGTGCTTACTTTTGTCTC3’	
18	5’ACCTTTTGCCAACACTAGCAT3’	52C
	5’TCCCAGCACCCACAGCC3’	
19	5’GGCAGACCCCTTCCTCAC3’	55C
	5’CCTGGGAGAGAGAAGCCTTT3’	
20	5’CTAGGTGTGAGTGCGAGTT3’	50C
	5’CAGCATTTGTCCCAGGT3’	
21	5’AATGGCTCAGATGCTGTT3’	50C
	5’GCTTGTGGTGAGTGGAGG3’	

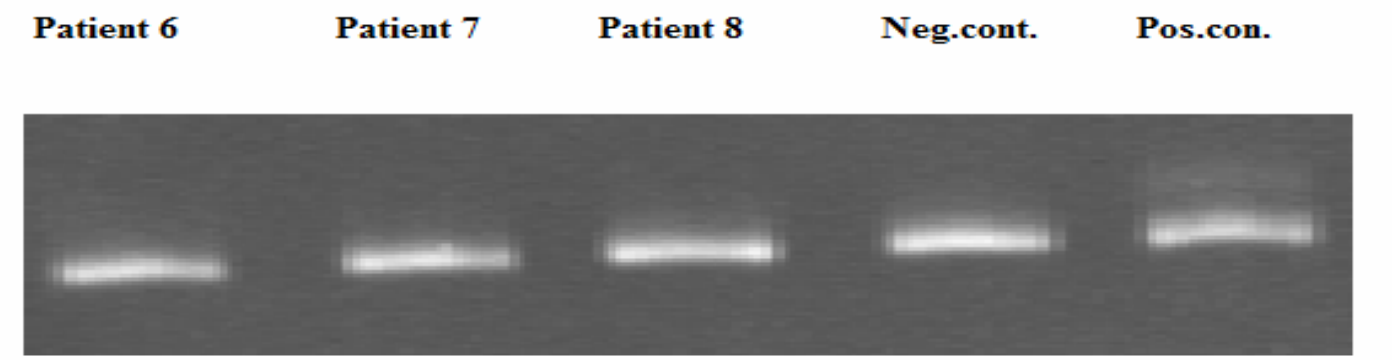


Fig 1 , CSGE results of exon 11 in three patients

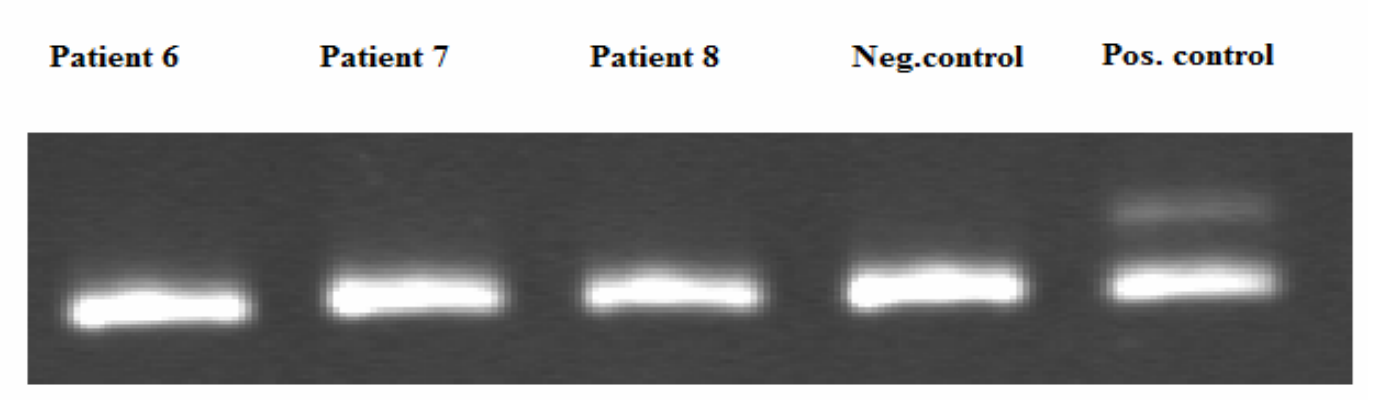


Fig 2 , CSGE results of exon 19 in three patients

Mutation confirmation (by sequencing)

As none of the samples tested, showed aberrant patterns (hetero duplex formation) on CSGE ; sequencing of the gene was not done.

Follow up of these patients

All patients diagnosed to have ACLD were treated with copper chelation , either D-Penicillamine or Zinc or both sequentially and followed up .

During each follow up the patient was examined clinically and investigated biochemically using liver function tests, 24 hr urinary copper,complete blood profile, prothrombin time and with calculation of MELD and CTP score.

Results

11 patients are included according to the diagnostic criteria for Atypical copper related liver disease (ACLD).

Characteristics of the patients with ACLD are shown in Table 2.

Table 2 : Patient characteristics

S.No	Age/ Sex	Age at the onset of illness	Date of illness	Date of first CMC visit	Date of treatment
1	22/ M	20	Sep 06	Sep 06	Sep 06
2	9 / M	9	Sep 07	Oct 07	Oct 07
3	18 / F	16	Jun 05	Apr 07	Apr 07
4	15 / M	15	Aug 07	Sep 07	Oct 07
5	9 / M	8	Mar 06	Mar 07	Mar07
6	15 / M	7	Oct 99	Oct 99	Dec 99
7	15 / F	7		Oct 99	Dec 99
8	13 / M	5		Oct 99	Dec 99
9	24 / F	20	Jun 03	Jun 07	Jul 07
10	23 / M	20	Jan 05	Jan 08	Mar 08
11	10 / F	7	Aug 04	Sep 04	Sep 04

11 ACLD patients from 9 families were studied. 8 of them were from Tamil Nadu, 2 were from Andhra Pradesh and 1from Kerala. In 1 family, all 3 children (including a twin pair) had ACLD, while in another family an elder sibling died of unexplained liver disease. There were 7 males and 4 females , median age was 16 , 9 – 24 years (median, range) . Although the age range was more than 20 years , these patients were included because of their disease presentation at less than 20 years of age.

Fig 3. Distribution of patients by state of origin

Clinical profile of the patients :

All patients were symptomatic during their presentation except 2 , who were asymptomatic siblings of a patient.

All patients presented with jaundice , 6 had hepatic decompensation in the form of ascites, one patient had hepatic encephalopathy at presentation, none had neurological involvement and coomb's negative heamolytic aneamia.

During the follow up two patients developed variceal bleeding and received endotherapy.

Patients	Asympto matic	Hepatic presentation					Neurological
		AH	CLD	Complications			
				Ascites	Coma	GIbleed	
1	-	-	√	√	-	√	-
2	-	√	-	-	-	-	-
3	-	√	√	√	√	-	-
4	-	√	-	-	-	-	-
5	-	-	√	√	-	-	-
6	-	-	√	√	-	√	-
7	√	-	-	-	-	-	-
8	√	-	-	-	-	-	-
9	-	-	√	√	-	-	-
10	-	-	√	√	√	-	-
11	-	-	-	-	-	-	-

Table 3 : Clinical presentation of patients

AH – Acute hepatitis
CLD – Chronic liver disease

Investigations :

Table 4 shows the investigative profile of these patients.

S.No	KF ring	Ceruloplasmin* (U/L)	24 hr urine copper@ (ug /24 hr)	Dry wt copper liver# (ug/g)	Serum Copper ug/dl$	ATP 7B mutation	Rhodanine Staining on liver biopsy	Rhodanine Staining on liver biopsy
1	Neg	175[a]	145	813	103	Neg	Present	Present
2	Neg	129[a]	1272		80		Present	Present
3	Neg	50[a]	78		56	Neg	Present	Present
4	Neg	81[a]	809	596	-		Absent	Absent
5	Neg	59[a]	21		-		Present	Present
6	Neg	92[a]	94		92	Neg	Present	Present
7	Neg	78[a]	87		120	Neg	Present	Present
8	Neg	176[a]	68		136	Neg	Present	Present
9	Pos	104[a]	189	449	-	Neg	Present	Present
10	Neg	379[b]	95	390	-		Present	Present
11	Neg	62[a]	122		40		Present	Present

* a – ceruloplasmin measured by O – dianisidine dihydrochloride method before 1st Nov 2007 normal range , 62 – 140 U/L

* b – ceruloplasmin measured by Ferroxidase method on and after 1st Nov 2007 , normal range, 200 -1100 U/L

@ 24 hr urine copper , normal range 40 -100 ug / 24 hr

Dry wt. liver copper , normal range < 250 ug / g

$ Serum copper level normal range 70- 170 ug /dl

Serum ceruloplasmin was normal in 9 patients, borderline low in 2 patients.KF ring was absent in all patients except 1. 24 hr urine copper was normal in 6 patients. Elevated 24 hr urine copper (152, 122 - 189µg) (median , range) was seen at baseline in 3 patients and in 2 patients although base line urine copper was normal, there was elevation after penicillamine challenge test . All 11 patients had copper deposits in liver biopsy. Rhodanine or orcein positivity was seen in all the patients except 1. Elevated dry weight copper was seen in 4 specimens tested (562, 320-813 µg/gm) (median, range).ATP7B mutations were absent in 6 patients tested.

Leipzig scoring : [85]

Leipzig scoring system for the diagnosis of wilsons disease is based on the following variables

ceruloplasmin

24 hr urine copper

KF ring

Hepatic copper content

Rhodanine stain on liver biopsy

coombs negative heamolytic aneamia

mutational analysis H1069Q (exon 14)

neurological symptoms

Liver copper (in absence of cholestasis)		**Serum Caeruloplasmin**	
Normal (<50µg/g)	-1	Normal(>0.2g/l)	0
<5xULN (50-250µg/g)	1	0.1-0.2g/l	1
>5xULN (>250µg/g)	2	<0.1g/l	2
Rhodanine Stain			
Absent	0		
Present	1		
Mutation Analysis		**Clinical symptoms and signs**	
2 chromosome mutations	4	KF rings	
1 chromosome mutation	1	Present	2
No mutations detected	0	Absent	0
Urinary copper (in absence of acute hepatitis)		**Neurological symptoms**	
Normal	0	Severe	2
1-2x ULN	1	Mild	1
>2x ULN	2	Absent	0
Normal but >5xULN after penicillamine	2	Coombs' negative haemolytic anemia	
		Present	1
		Absent	0

Table 5 Leipzig scoring

A total score of 4 is considered as Wilsons disease

2-3 likely to have Wilsons disease

0-1 unlikely to have Wilsons disease

Table 6: Leipzig scoring system for ACLD patients

S.No	Leipzig score
1	**4**
2	**3**
3	**2**
4	**4**
5	**2**
6	**2**
7	**1**
8	**1**
9	**4**
10	**3**
11	**2**

Liver histology :

Mallory's hyaline, pericellular fibrosis , hepatocyte ballooning degeneration, absence of fatty change , moderate to severe inflammatory activity , bridging fibrosis and significant stainable copper deposition were the predominant histological features observed on the liver biopsy specimens.

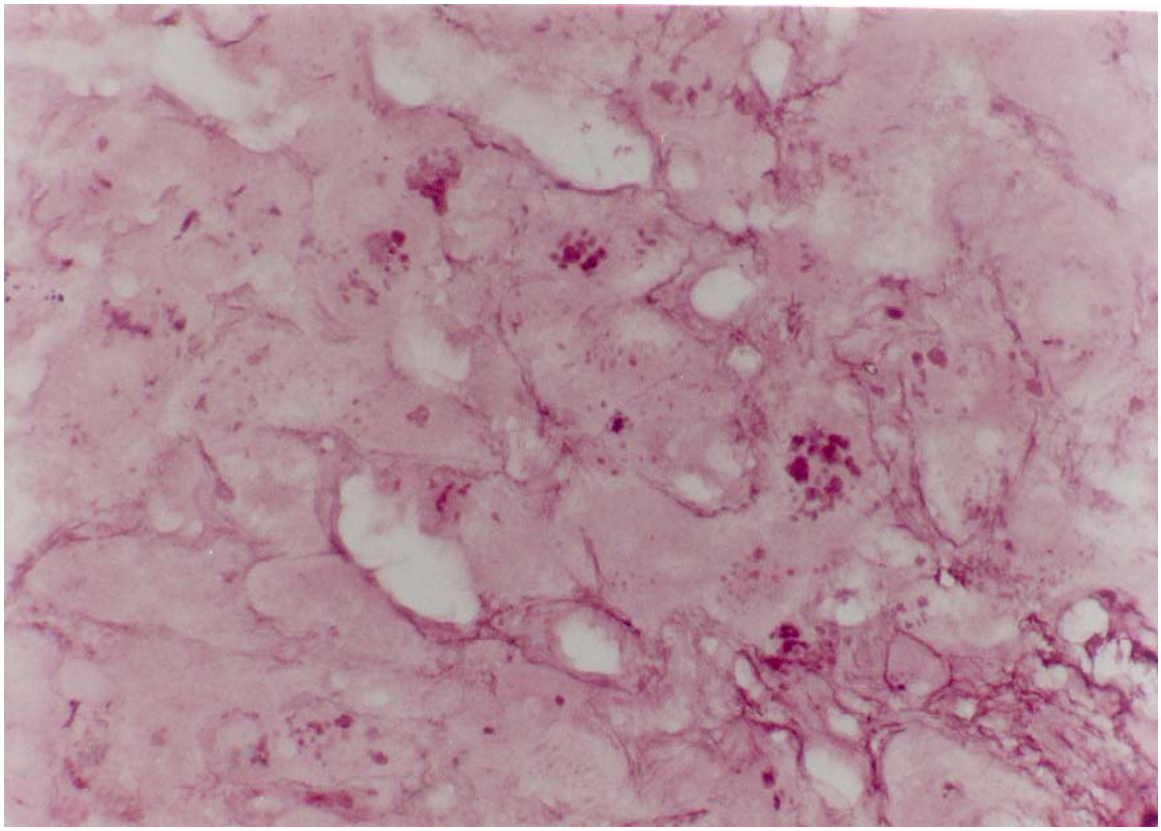


Fig 4 Orcein stain shows fine granules of copper associated protein in periportal hepatocytes (Orcein X 400) in patient 6

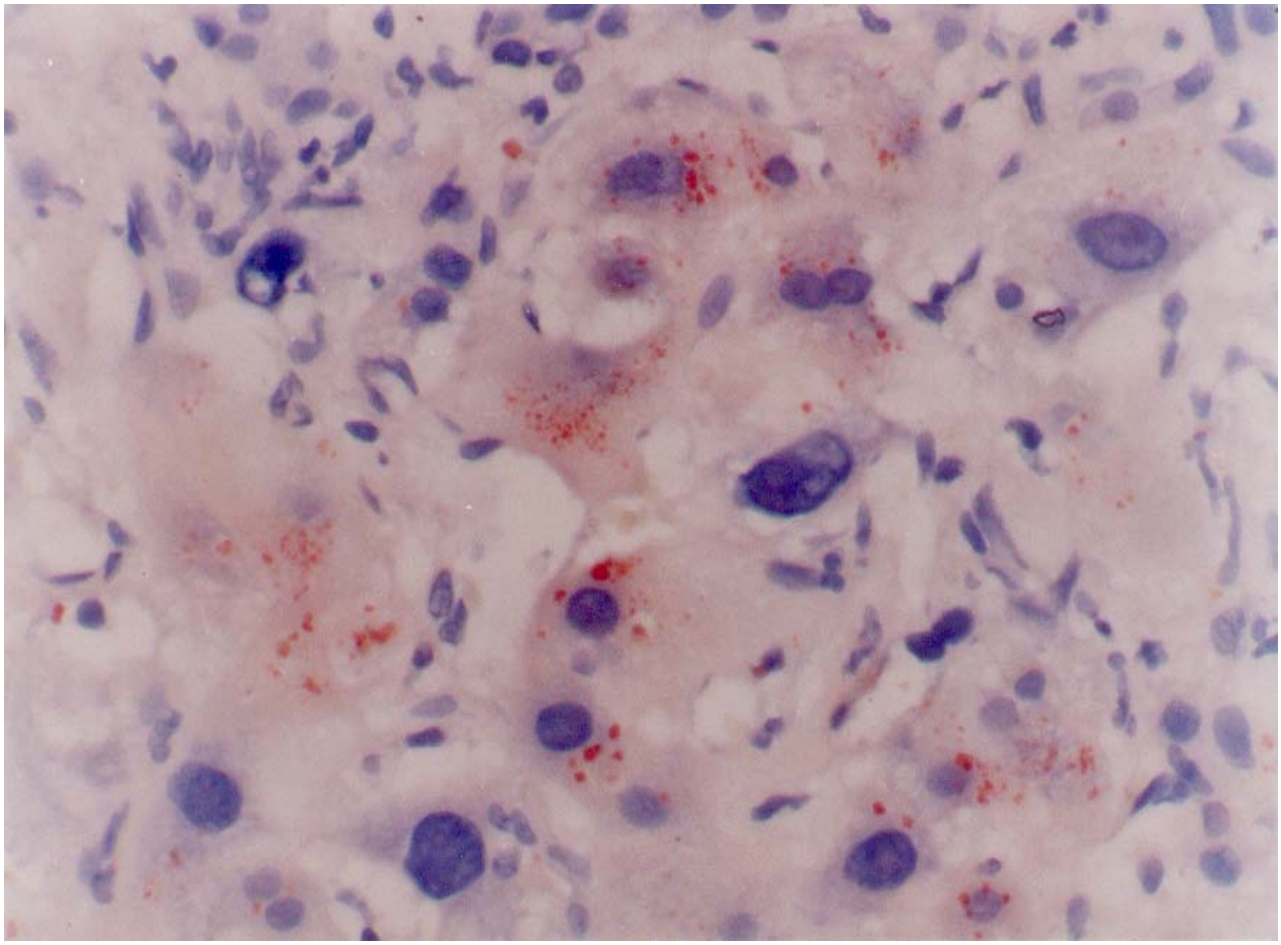


Fig 5 Rhodamine stain shows granular deposits of copper in periportal hepatocytes (Rhodamine X 400) in patient 6

Table 7 shows the histological features of all the patients.

Patient	Fattychange	Pericellular fibrosis	Mallory's hyaline	Ballooning degeneration	CAP	Presence of cirrhosis
1	-	-	✓	✓	**2+**	✓
2	-	✓	✓	✓	**2+**	✓
3	-	✓	✓	-	**1+**	✓
4	-	-	-	✓	**2+**	✓
5	-	-	-	-	**2+**	✓
6	-	-	-	✓	**2+**	✓
7	-	-	-	-	**1+**	-
8	-	-	-	-	**1+**	-
9	-	✓	✓	✓	**2+**	✓
10	-	-	-	-	**2+**	-
11	-	✓	✓	-	**2+**	-

Table 7 : Liver biopsy findings in all patients

In three patients even though the copper staining was 1+ they were included in the study because of strong family history and normal copper studies.

Drinking water copper content :

Drinking water copper was measured in all the samples collected from the residences of the patients right from birth. Those samples were collected and processed as described earlier using flame atomic absorption spectrometry based upon cloud point extraction. 17 water samples were obtained from different sites of residence from the 9 families. Copper content in drinking water was normal in all samples (2.32 µg/L, 0.32-8.9) (median, range).

Dietary history for copper intake :

All ACLD patients were asked about history of cooking in brass vessels. History of storing drinking water in brass vessels was obtained in 1 family, cooking in brass vessels in none.

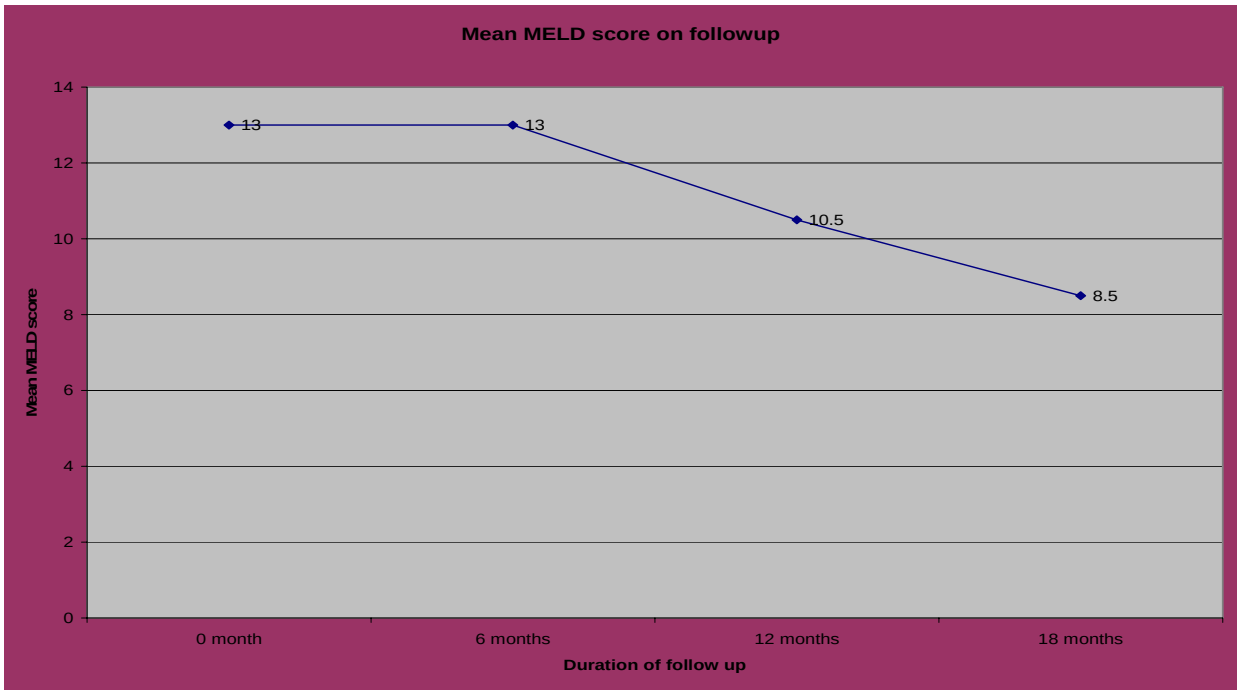
Table 8: Drinking water copper levels

S.No	Drinking Water copper (ug/l)
1	**2.86**
2	**2.36**
3	**4.04**
4	**1.08**
5	**0.32**
6	**0.7**
7	**0.7**
8	**0.7**
9	**1.22**
10	**2.68**
11	**8.9**

Follow up of the patients :

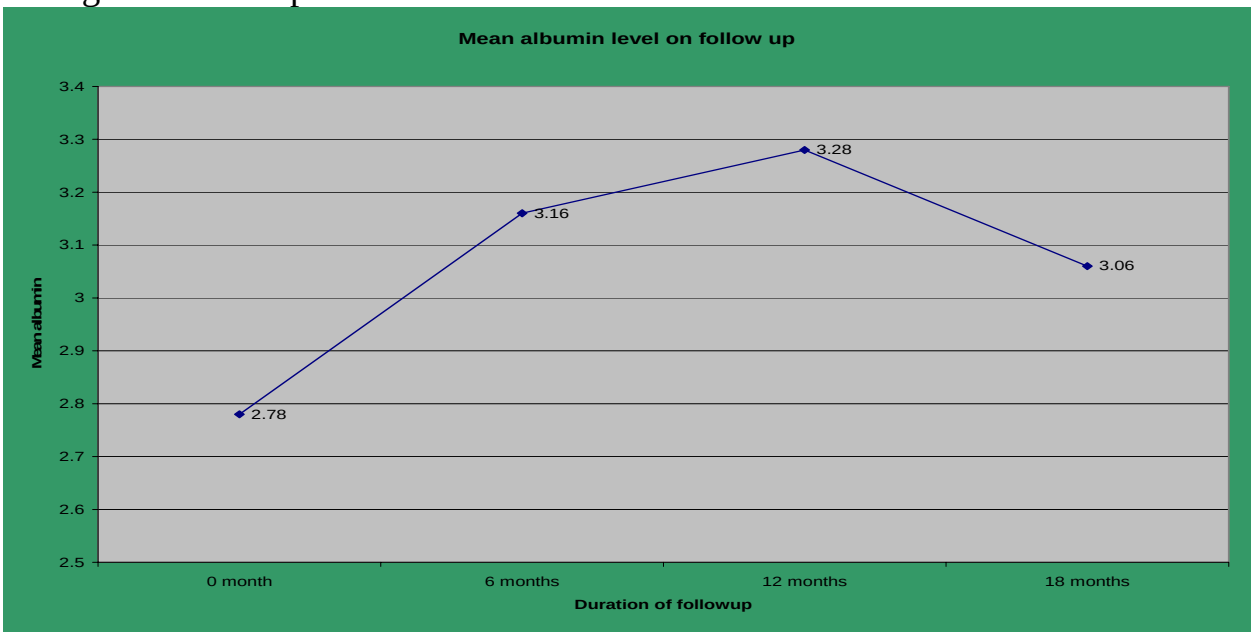
All ACC patients were treated with copper chelation either with D-Penicillamine or Zinc or both sequentially. They were followed up for a period of 41 months , 3 – 108 months (median, range)

During the follow up Mean MELD score was decreased from 13 at baseline to 8.5 at median follow up of 41 months.



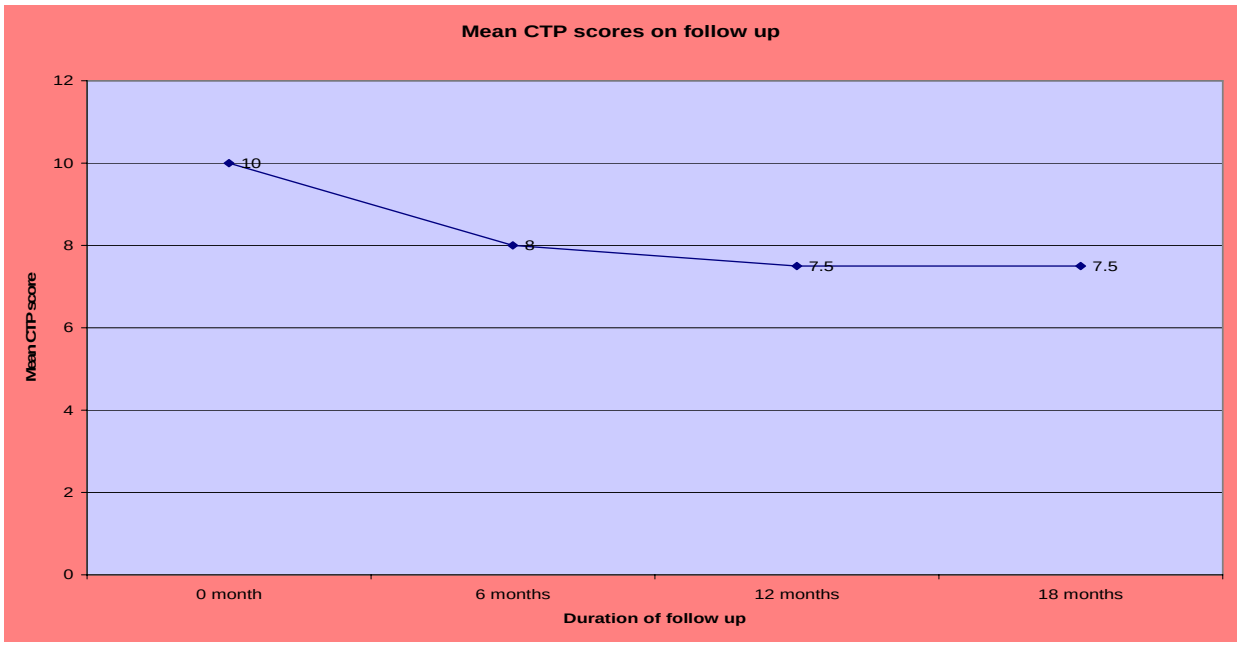
Graph 1 showing Mean MELD score on followup of ACLD patients

Mean improvement in albumin level was from 2.78gm/dl at baseline to 3.06gm/dl during the follow up



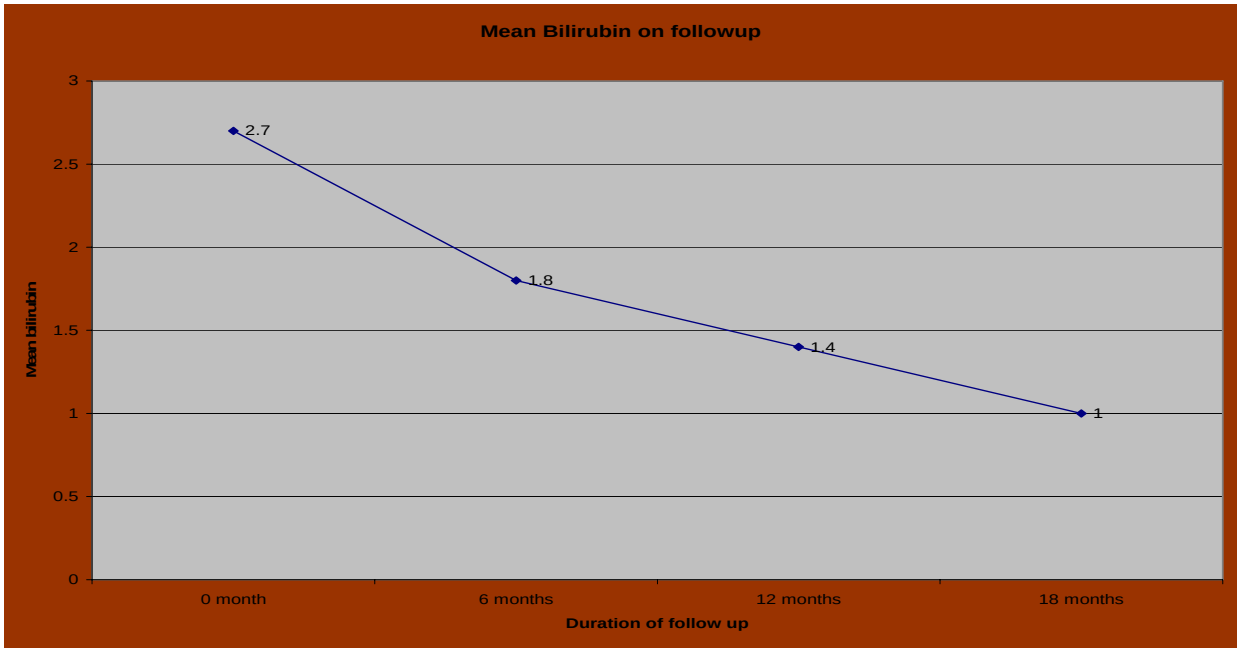
Graph 2 showing Mean Albumin levels on followup

During the follow up, Mean CTP score was fallen down from 10 to 7.5 with median duration of 41 months.

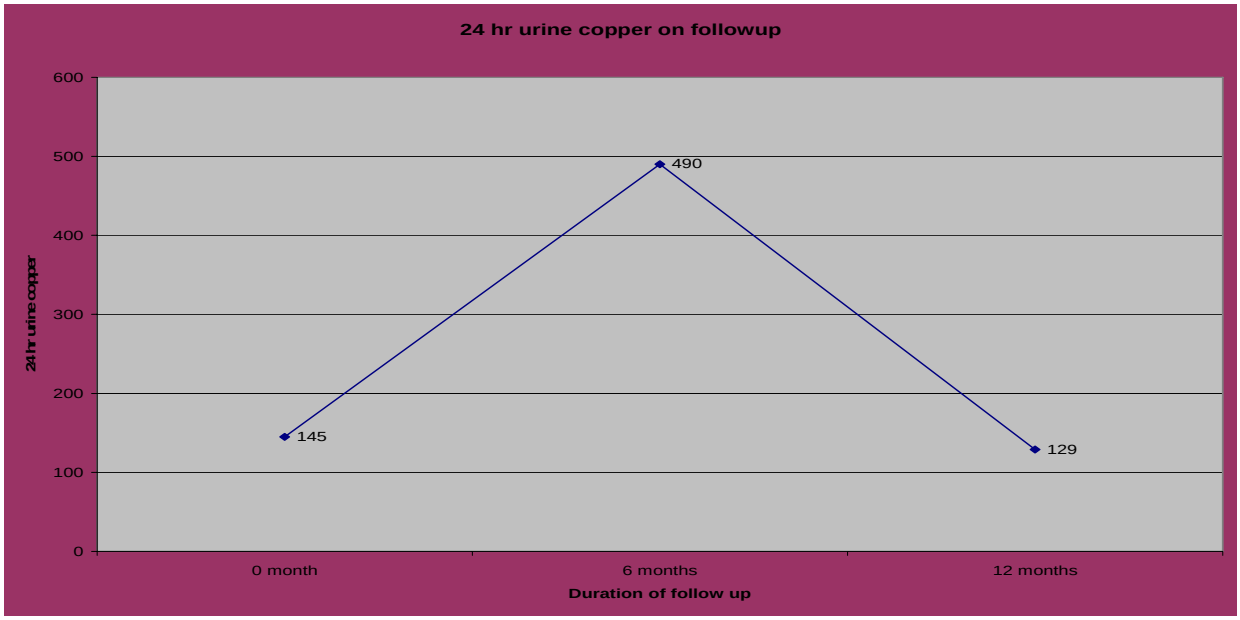


Graph 3 showing Mean CTP score on followup

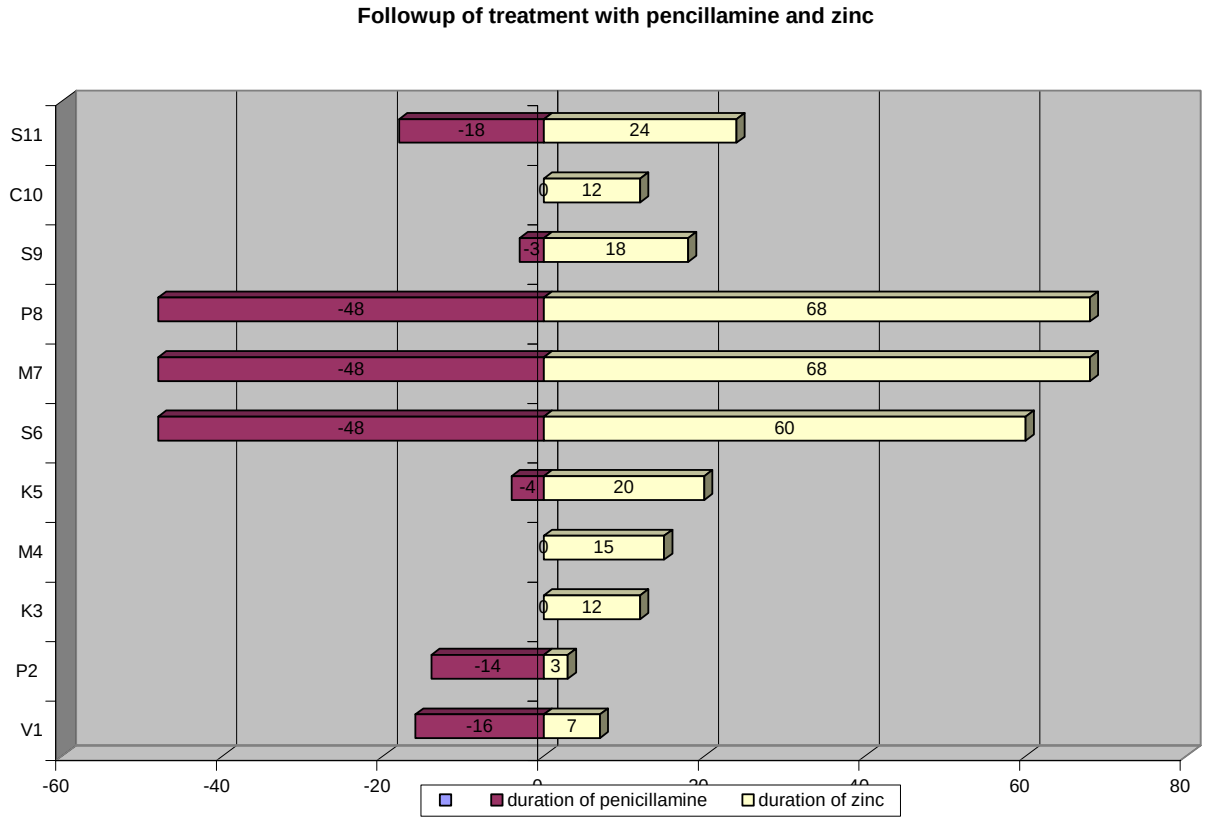
Mean bilirubin and 24 hr urine copper levels were decreased from 2.7mg /dl and 145ug/24hrs at baseline to 1mg/dl and 129ug/24hrs during 41median months of follow up respectively.



Graph 4 showing Mean Bilirubin on followup



Graph 5 showing Mean 24 hr urine copper levels on followup



Graph 6 showing duration of treatment with copper chelation

Survival:

During the follow up period Patient 1 had recurrent variceal bleed with hepatic decompensation ultimately succumbed to death at his home town. Patient 3 expired with end stage liver disease with persistent jaundice, recurrent encephalopathy and ascites , was on transplant list for the same. Patient 6 expired at his home town unrelated to liver disease. All the patients received Penicillamine initially except 3 who were started on zinc directly. Penicillamine was given for mean duration of 18 months followed by zinc for a mean duration of 27 months.

Discussion

This thesis reports 11 patients with atypical copper related liver disease – an example of hepatic copper toxicosis, which does not fit either into typical Wilsons' disease or Indian Childhood Cirrhosis.

The hallmark of this group of patients is presence of increased copper on liver biopsy on staining and by dry weight copper estimation, with normal serum ceruloplasmin and absence of KF rings in the eye. Three of the 11 patients had a score of 4 by Leipzig criteria (which is diagnostic of Wilson's disease). However, the significantly increased liver copper on staining and dry weight estimation accounted for a score of 2, in the 3 patients with Leipzig score of 4.

In this study the clinical presentation and treatment outcome of ACLD was described. In addition to that it was hypothesized that whether these patients were taking excessive amounts of copper in the form of cooking in brass vessels or increased levels of copper in the drinking water.

The clinical presentation was no different phenotypically from any other form of liver disease or Wilsons'disease as far as hepatic presentation is concerned. All patients presented with jaundice except two who were asymptomatic siblings. 6 patients had hepatic decompensation . All were treated with copper chelation with

either D-penicillamine or Zinc or both sequentially. All except 3 had improvement in clinical and laboratory parameters. Drinking water that was collected from the patients' residences right from the birth for copper content showed normal water copper levels (2.32 µg/L, 0.32-8.9) (median, range). The action level for water copper level is 1300ug/L. The drinking water copper levels collected from the patients' residences were well below the action level stating that copper accumulation in the liver is not due to increased exogenous intake.

Further more, though phenotype was no different from WD, the laboratory parameters were not correlating with WD. ATP 7B mutations screening using Conformation sensitive gel electrophoresis in 6 patients tested were shown negative .

The histological features that can distinguish ACLD from WD and ICC are depicted in table 8

	Fatty change	Peri cellular fibrosis	Mallory hyaline
ACLD	no fatty change	mild	abundant
WD	glycogenated cells	nil	present
ICC	no fatty change	creeping fibrosis	abundant

Table 9 Differentiation of ACLD from WD and ICC

The hepatic copper accumulation in Wilson's disease is now thought to be due to defective functioning of ATP 7B protein (the product of the Wilson's disease gene or ATP 7B gene). ATP 7B protein is postulated to facilitate copper excretion across the canalicular membrane into the biliary canaliculus. In Indian Childhood Cirrhosis, contaminaiton of milk was thought to be the cause of increased copper content in the liver. The hepatic copper deposition in ACLD may probably due to a defect in the copper metabolism other than WD. It may be a genetic disorder as was seen occurring in siblings in two families or it may be expressing in conjunction with an unknown environmental toxin.

Earlier studies by Muller et al [81] provided strong evidence that Idiopathic copper toxicosis or endemic infantile Tyrolean cirrhosis is an ecogenetic disorder affecting a genetically predisposed fraction of the population, and requiring both homozygosity for a defective gene and high levels of exogenous copper for disease manifestation. The likelihood that the abnormal gene persists in healthy heterozygous siblings should enable its identification, thereby proving that we are dealing with a genetic disorder of copper metabolism.

ACLD has to be kept as a differential diagnosis while one is dealing with any of the copper related liver diseases. The diagnosis was established largely by liver biopsy in the above patients underscores the importance of liver biopsy in investigating cryptogenic liver disease. The diagnosis would have been missed in all these patients if liver biopsy would not have been performed and hence copper chelation.

In this study there was no exogenous copper intake that was demonstrated. With regard to the genetic aspects of the disease ATP 7B gene mutations was not found indicating that there may be unknown defects in other part of the genome that is responsible for the expression of the disease.

Further studies that look into the copper kinetics, characterization of ATP 7 B protein, other copper transporting molecules and any other defect in the copper metabolism needs to be performed

Conclusion

The conclusions drawn from this study are

1. The 11 patients have features of hepatic copper overload not fitting into either WD or ICC
2. This condition is different from WD by not showing neurological involvement and coombs negative heamolytic aneamia with normal ceruloplasmin, and negative for Kayser Fleisher rings and ATP 7 B mutations.
3. This condition is different from ICC by the occurrence of disease after 5 years with no dietary history of copper intake and histology different from ICC.
4. The treatment outcome of ACLD patients with either Penicillamine or Zinc or both sequentially is good.
5. There was no evidence for increased copper intake in drinking water in ACLD patients studied.
6. Further studies into causation of ACLD are needed .

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APPENDIX I - PROFORMA

Case Number

Name

Age

Sex

Hospital number

Address

Date of illness

Date of CMC Consultation

Date of diagnosis

Final diagnosis and basis of diagnosis

Date of starting of treatment

Clinical details

Jaundice:

Cholestatic or non cholestatic

Duration

Prodrome

Associated with hepatic decompensation

Abdominal distension :

Uniform

Painless or painful

Intermittent or progressive

Pedal oedema

Altered sensorium

Other complications

Oliguria

Fever

Weight loss and loss of apetite

Drug intake

Dietary history

Family history

Personal history

Social history

Treatment history

Physical examination

Height : weight : BMI :
Pallor
Icterus
Clubbing
Cyanosis
Pedal oedema
Lymphadenopathy

Systemic examination

Abdominal :

Respiratory :

Cardiovascular :

Others:

Investigations

1. Complete blood profile
   Liver function tests
   Prothrombin time, Activated partial thromboplastin time
   Serum creatinine and electrolytes

2. Radiological and endoscopy tests
   Chest x ray PA view
   USG abdomen
   Oesophagogastroduodenoscopy

3. Etiological work up for liver disease
   Blood borne virus infection
   Antinuclear antibody
   Serum immunoglobulins
   Total core antibody

4. Wilsons' Disease work up
   Serum ceruloplasmin
   24 hr urinary copper
   Hepatic copper estimation
   KF ring
   ATP 7B mutation analysis

5. liver biopsy (percutaneous / transjugular)

6. Drinking water copper level

APPENDIX – II MASTER SHEET

S No	Patient name	Hospital no.	Age	Sex	Date of illness	Date of CMC	Initiation of treatment	KF ring present-2	KF ring absent-0
1	Vinoth kumar	848905c	22	M	Sep-06	Sep-06	Sep-06		Neg
2	Prem kumar	110163d	9	M	Sep-07	Oct-07	Oct-07		neg
3	Krystal	000929d	18	F	Jun-05	Apr-07	Apr-07		Neg
4	Mubthi	080334d	15	M	Aug-07	Sep-07	Oct-07		Neg
5	Krishna raj	993332c	9	M	Mar-06	Mar-07	Mar-07		neg
6	siva kumar	778355b	15	M	Oct-99	Oct-99	Dec-99		neg
7	monica	795562b	14	F		Oct-99	Dec-99		neg
8	purushothaman	792595b	13	M		Oct-99	Dec-99		Neg
9	smitha rajan	061183d	24	F	Jun-03	Jun-07	Jul-07	positive	
10	chenna kesava	172104d	23	M	Jan-05	Jan-08	Mar-08		Neg
11	sobika	278652c	9	F	Aug-04	Sep-04	Sep-04		Neg

neurological symptoms			Coomb's negative heamolytic aneamia		ceruloplasmin		
severe-2	mild-1	absent-0	present-1	absent-0	normal-0	half-1	less-2
		abs		abs	175		
		abs		abs	129		
		abs		abs		50	
		abs		abs	81		
		abs		abs		59	
		abs		abs	92		
		abs		abs	78		
		abs		abs	176		
		abs		abs	104		
		abs		abs	379 new		
		abs		abs	62		

		urine copper			liver copper			rhodanine stain	
normal-0	1-2x ULN	>2x ULN	>5ULN,post pencillamine		<50ug --1	50-250 - 1	>250 - 2	absent - 0	present - 1
	145						813ug /g		present
				1272					present
78									present
111				809			596 ug/g	absent	
21									present
94									present
87									present
68									present
	189						449ug/g		present
95							390ug/g		present
	122								present

mutation analysis			water copper level
2 muta - 4	1 muta - 2	no muta- 0	
		neg	2.86
			2.36
		neg	4.04
			1.08
			0.32
		neg	0.7
		neg	0.7
		neg	0.7
		neg	1.2
			2.68
			8.9
			2.32

Leipzig score	MELD						
	base line	6 months	1 year	1.5years	2years	2.5years	3years
4	10	13	14	13	11		
3	18	11	10				
2	16	18	21	26			
4	13	15	11				
2	15	8	7	7			
2	18	16	17	13	16	14	8
1	6	6	6	6			
1	12	7	6	6			
4	13	10	9	8			
3	17	15					
2	7	13	12	9	8		
Median	13	13	10.5	8.5			
Mean	13.18	12	11.3	11			

	Albumin					
base line	6 months	1 year	1.5years	2years	2.5years	3years
3.7	3.4	2.3	2.2	1		
2.3	2.9	3.4				
2.4	2.5	1.9	2.6			
2.1	3.2	3.9				
1.6	3.1	2.9	2.4			
2.6	2.9	3.9	2.3	2.9	2.8	3
3.7	4.1	4.2	4.4			
2.9	3.9	4.4	4.4			
2	2.4	2.6	2.6			
2.3	2.8					
5	3.6	3.3	3.6	4.7		
2.4	3.1	3.35	2.6			
2.78	3.16	3.28	3.06			

CTP Score

base line	6 months	1 year	1.5years	2years	2.5years	3years
6	7	10	10	11		
11	8	6				
10	11	12	13			
10	9	6				
10	7	7	7			
11	10	9	9	9	9	7
5	5	5	5	5		
9	5	5	5			
10	8	8	8			
12	9					
5	7	8	6	5		
10	8	7.5	7.5			
9	7.8	7.6	7.8			

bilirubin mg/dl

base line	6 months	1 year	1.5years	2years	2.5years	3years
1.9	3.3	3.8	3.2	2.4		
4.1	1.7	1.4				
3	3.7	9.4	42.1			
1.5	1.9	0.9				
3	0.6	0.6	1			
5.3	4	5.3	2.5	3	2.6	1.7
0.3	0.6	0.7	0.5			
2.7	0.9	0.9	0.9			
3.2	1.5	1.5	1.3			
2.7	2.5					
0.8	2.3	1.6	1	0.6		
2.7	1.8	1.4	1			

		24hr urine copper ug/24 hr				
base line	6 months	1 year	1.5years	2years	2.5years	3years
145						
1272	368					
78						
809		73				
21	81					
150	672					
87	490	154	48			
48	705	104	38			
189	72					
95						
256	663	411	64	77		

Legends

Graph 1 showing Mean MELD score on followup of ACLD patients

Graph 2 showing Mean Albumin levels on followup

Graph 3 showing Mean CTP score on followup

Graph 4 showing Mean Bilirubin on followup

Graph 5 showing Mean 24 hr urine copper levels on followup

Graph 6 showing duration of treatment with copper chelation